TRANSGENIC CANCER MODELS IN FISH

BACKGROUND OF THE INVENTION

The present invention relates to transgenic animals. Specifically, the invention relates to transgenic fish and methods for their use.

Animal models of disease states play an important role in identifying the underlying biochemical mechanisms of particular diseases, as well as discovering therapeutic agents to eradicate the disease or otherwise lessen its symptoms. For example, rabbit models of familial hypercholesterolemia, rat models of non-insulin-dependent diabetes mellitus, mouse models of cancer and hamster models for spontaneous atrial thrombosis are known. Additionally, animal models for genetic diseases have arisen spontaneously in a variety of species, including mice, cats and dogs. Working with such large animals poses several drawbacks.

For example, many of the animals used in such models are relatively large vertebrates which take up a large amount of research space, are costly to feed and otherwise maintain, have slow reproductive cycles, produce relatively few offspring at one time, and cannot effectively mimic all desired disease states. Researchers have attempted to obtain animal models that solve some of these problems, but have not yet obtained such animal models for all desired diseases. For example, transgenic fish models of premalignant and non-neoplastic or non-malignant hyperproliferative disorders (*e.g.,* inflammation and retinopathy) would also be useful. Additionally, although fish have been utilized to detect mutagens in aquatic environments, there are currently no transgenic fish models that develop any cancers relevant in human cancer research, including, for example, human T-cell leukemias, non-Hodgkin's lymphoma, high-grade astrocytoma, rhabdomyosarcoma, neuroblastoma, neuorendocrine carcinoma, pancreatic carcinoma, ovarian carcinoma, testicular carcinoma, stomach cancer, colon cancer, renal cancer, melanoma and acute or chronic myeloid leukemia.

Human T-cell leukemias can arise from oncogenes activated by specific chromosomal translocations involving T-cell receptor genes. In particular, T-cell acute lymphoblastic leukemia is a malignant disease of thymocytes, accounting for about 10% to about 15% of pediatric and about 25% of adult acute lymphoblastic leukemia cases. Although some therapies are available for T-cell acute lymphoblastic leukemia, they are not as effective as desired.

Follicular center cell non-Hodgkin's lymphomas are a common and generally indolent type of B-cell lymphoma that occurs almost exclusively in adults, and 80% of follicular center cell lymphomas have a t(14;18) chromosomal translocation. Molecular analysis of the breakpoints of the 14;18 translocation identified *BCL2* as the gene on chromosome 18 that is overexpressed due to its translocation into the *IgH* locus on chromosome 14. Functional studies revealed that BCL2 defines a new class of proto-oncogene products that act to prolong cell survival, rather than through more typical effects on cell differentiation or proliferation. It has since been learned that BCL2 is a member of a large family of highly conserved proteins that either inhibit or promote apoptosis. In addition, BCL2 and it's pro-survival relatives may be important for the aberrant survival of many human cancers, not just those with overexpression of BCL2 due to the t(14;18).

High-grade astrocytomas are among the most common and devastating adult brain tumors, spreading so rapidly that patients seldom survive more than 9-12 months. Despite progress in surgical, radiation and chemotherapy technologies, there has been little improvement in the outcome of patients with astrocytoma over the last twenty years. Clearly, novel approaches are needed to better understand the biological basis of this disease before effective therapies can be developed.

Rhabdomyosarcomas are a heterogeneous group of malignant tumors of skeletal muscle progenitors and are the most common soft-tissue sarcoma in children of 15 years or younger. Rhabdomyosarcoma consists of two histologic subtypes, alveolar and embryonal, each characterized by the misexpression of

different subsets of genes. The aggressive nature of these tumors makes their effective treatment particularly difficult. While rhabdomyosarcomas can be observed in genetically engineered, mammalian disease models, they are often associated with other tumor types. While informative, a more specific model of rhabdomyosarcoma is necessary to elucidate its molecular basis and to identify novel genes that may ultimately be used as targets for the development of novel therapeutic strategies.

The mutations and gene rearrangements commonly seen in acute myeloid leukemias typically result from a chromosomal translocations such as the t(8;21) or t(15;17), generate chimeric oncoproteins by fusing one or two transcription factors (Look, 1997). However, these alterations are not sufficient to explain the induction of acute leukemia. Additional animal models are needed, for example, to permit the unbiased detection of mutations in many potentially novel genes that lead to leukemia, which is currently not possible in other mammalian models.

Many of the underlying mechanisms that lead to neuroblastoma, neuorendocrine carcinoma, pancreatic carcinoma, ovarian carcinoma, testicular carcinoma, stomach cancer, colon cancer, renal cancer, melanoma and acute or chronic myeloid leukemia have yet to be fully understood. Identifying the genes mutated in these diseases will lead to new insights into cancer as a whole. Additionally, using a vertebrate model system in which genetic or chemical suppressors can be identified that inhibit or delay disease progression, or sensitivity to chemotherapy or radiation-induced programmed cell death, will be necessary to identify new drug targets for the development of targeted chemotherapies. For example, a model system is needed, which does not require an *a priori* knowledge of the specific target. Target elucidation may be accomplished after the modulating target drug or agent is demonstrated safe and effective, which, thus, saves both time and expense in the drug discovery process.

A further understanding of the cellular and molecular genetic features of various disease states such as the cancers listed above are needed. An appropriate animal model would be invaluable to elucidate the multistep process of genetic mutations, as well as to develop more effective drugs. The present invention addresses these needs.

SUMMARY OF THE INVENTION

It has been discovered that transgenic fish may be produced that can advantageously be utilized as a fish model of mammalian disease, including cancers, and particularly human cancers. Accordingly, transgenic fish are provided, as are methods of their production and use.

In one aspect of the invention, a transgenic fish is provided whose genome has stably-integrated therein an oncogene operably linked to a promoter. In one form, transgenic fish is provided whose genome has stably integrated therein a ubiquitous gene promoter, a reporter gene comprising a strong transcription stop-site, and an oncogene, wherein the reporter gene is flanked by site-specific recombinase recognition sites. Preferably the promoter is an organ- or tissue-specific promoter. In one embodiment, the promoter is a lymphoid-specific promoter. In certain forms of the invention, the oncogene induces a leukemia or lymphoma, including a T-cell lymphoma or a T-cell acute lymphoblastic leukemia. In other embodiments, the oncogene induces non-Hodgkin's lymphoma, high-grade astrocytoma, rhabdomyosarcoma, neuroblastoma, neuorendocrine carcinoma, pancreatic carcinoma, ovarian carcinoma, testicular carcinoma, stomach cancer, colon cancer, renal cancer melanoma and acute or chronic myeloid leukemia.

In a second aspect of the invention, methods of making a transgenic fish are provided. In one form, a method includes introducing nucleic acid into a fertilized fish embryo, wherein the nucleic acid comprises an oncogene operably linked to a promoter, and developing the fish embryo into a transgenic fish. In another form, a method includes introducing nucleic acid into a unfertilized fish egg, wherein the nucleic acid comprises an oncogene operably linked to a promoter, fertilizing the fish egg, and developing the fish embryo into a transgenic fish.

In another aspect of the invention, methods of screening for drugs or agents that modulate (*e.g.,* enhance or suppress) oncogene-mediated neoplastic or hyperplastic transformation. In one embodiment, a method includes (a) contacting or otherwise exposing a transgenic fish (*e.g.,* an adult transgenic fish or a transgenic fish embryo) to a test drug or agent, wherein the transgenic fish has a genome that has stably-integrated therein an oncogene operably linked to a promoter; (b) determining if the test drug or agent modulates (*e.g.,* enhances or suppresses) oncogene-mediated neoplastic or hyperplastic transformation; and (c) classifying the test drug or agent as an drug or agent that modulates oncogene-mediated neoplastic or hyperplastic transformation if the test drug or agent suppresses or enhances oncogene-mediated neoplastic or hyperplastic transformation.

In another embodiment, a method includes (a) contacting or otherwise exposing a transgenic fish (*e.g.,* an adult transgenic fish or a transgenic fish embryo) to a test drug or agent, wherein the transgenic fish has a genome that has stably integrated therein a ubiquitous gene promoter, a reporter gene comprising a strong transcription stop-site, and an oncogene, and wherein the reporter gene is flanked by site-specific recombinase recognition sites; (b) determining if the test drug or agent modulates oncogene-mediated neoplastic or hyperplastic transformation; and (c) classifying the test drug or agent that modulates oncogene-mediated neoplastic or hyperplastic transformation.

In yet another aspect of the invention, methods of screening for drugs or agents that modulate the sensitivity of transgenic cells to treatment with radiation or chemotherapy are provided. A method includes (a) contacting or otherwise exposing a transgenic fish (*e.g.,* an adult transgenic fish or a transgenic fish embryo) to a test drug or agent, wherein the transgenic fish has a genome that has stably-integrated therein an oncogene operably linked to a promoter; (b) determining if the test drug or agent modulates (*e.g.,* suppresses or enhances) sensitivity to radiation- or chemotherapy-induced programmed cell death; and (c) classifying the test drug or agent as an drug or agent that modulates sensitivity to

radiation- or chemotherapy-induced programmed cell death if the test drug or agent suppresses or enhances sensitivity to radiation- or chemotherapy-induced programmed cell death.

In another embodiment, a method includes (a) contacting or otherwise exposing a transgenic fish (*e.g.,* an adult transgenic fish or a transgenic fish embryo) to a test drug or agent, wherein the transgenic fish has a genome that has stably integrated therein a ubiquitous gene promoter, a reporter gene comprising a strong transcription stop-site, and an oncogene, and wherein the reporter gene is flanked by site-specific recombinase recognition sites; (b) determining if the test drug or agent the sensitivity of transgenic cells to treatment with radiation or chemotherapy; and (c) classifying the test drug or agent that modulates the sensitivity of transgenic cells to treatment with radiation or chemotherapy if the test drug or agent suppresses or enhances sensitivity to radiation- or chemotherapy-induced programmed cell death.

In another aspect of the invention, methods of identifying mutations that modulate oncogene-mediated neoplastic or hyperplasic transformation or sensitivity to radiation- or chemotherapy-induced programmed cell death are provided. In one embodiment, a method includes (a) mutagenizing a transgenic fish whose genome has stably-integrated therein an oncogene operably linked to a promoter; (b) mating the transgenic fish with a non-mutagenized fish to produce F_1 offspring; (c) obtaining eggs from the F_1 offspring; (d) fertilizing the eggs with inactivated sperm to produce F_2 offspring; (e) determining the presence and extent of neoplasia or hyperplasia or sensitivity to radiation- or chemotherapy-induced programmed cell death in the F_2 offspring; and (f) identifying the modulator (*e.g.,* enhancer or suppressor) mutation in the F_1 offspring.

In another embodiment, a method includes (a) mutagenizing a transgenic fish whose genome has stably integrated therein a ubiquitous gene promoter, a reporter gene comprising a strong transcription stop-site, and an oncogene, and

wherein the reporter gene is flanked by site-specific recombinase recognition sites; (b) mating the transgenic fish with a non-mutagenized fish to produce F_1 offspring; (c) obtaining eggs from the F_1 offspring; (d) fertilizing the eggs with inactivated sperm to produce F_2 offspring; (e) determining the presence and extent of neoplasia or hyperplasia or sensitivity to radiation- or chemotherapy-induced programmed cell death in the F_2 offspring; and (f) identifying the mutation that modulates oncogene-mediated neoplastic or hyperplastic transformation or that modulates sensitivity to radiation- or chemotherapy-induced programmed cell death.

In other embodiments, a method includes (a) mutagenizing a transgenic first fish, wherein said transgenic first fish is homozygous for a transgene; (b) mating said transgenic first fish with a non-mutagenized second fish, to produce F_1 fish, wherein said non-mutagenized second fish is homozygous for the transgene; (c) obtaining eggs from female F_1 fish; (d) fertilizing said eggs with inactivated sperm; (e) subjecting said fertilized eggs to early pressure to produce gynogenetic diploid F_2 fish; (f) examining the rate of onset and/or extent of organ or tissue-specific neoplasia or hyperplasia to identify F_1 fish that are heterozygous for inactivation of a tumor suppressor gene; and (g) optionally out-crossing the F_1 female fish to identify the mutation.

In yet another aspect of the invention, methods of co-expressing more than one mammalian oncogene in a transgenic fish are provided. In one embodiment, a method includes: (a) producing a first transgenic fish whose genome has stably-integrated therein a first mammalian oncogene operably linked to a first promoter; (b) producing a second transgenic fish whose genome has stably-integrated therein a second mammalian oncogene operably linked to a second promoter, wherein said first mammalian oncogene and said second mammalian oncogene are different; and (c) mating said first transgenic fish with said second transgenic fish to produce offspring, which co-express said different mammalian oncogenes.

In another embodiment, a method includes (a) producing a transgenic fish whose genome has stably integrated therein a ubiquitous gene promoter, a reporter gene comprising a strong transcription stop-site, and a first mammalian oncogene, wherein the reporter gene is flanked by site-specific recombinase recognition sites; (b) producing a second transgenic fish whose genome has stably-integrated therein a second mammalian oncogene operably linked to a second promoter; wherein said first mammalian oncogene and said second mammalian oncogene are different; and (c) mating said first transgenic fish with said second transgenic fish to produce offspring, which coexpress said different mammalian oncogenes.

In another aspect of the invention, methods of making a stable transgenic fish model of human cancers are provided, wherein an oncogene can be regulated in any tissue by using regulatable site-specific recombinases. In one form, a method includes (a) producing a first transgenic fish whose genome has stably integrated therein a cassette comprising a ubiquitous gene promoter, a reporter gene comprising a strong transcription stop-site and flanked by site-specific recombinase recognition sites, and an oncogene; (b) producing a second transgenic fish whose genome has stably integrated therein a site-specific recombinases operably linked to a heat shock promoter; (c) mating said first transgenic fish with said second transgenic fish to produce F_1 progeny fish; (d) laser-activating cells of said F_1 progeny fish, whereby said laser activation causes recombination and excision of said reporter gene and juxtaposition of said ubiquitous gene promoter adjacent to said oncogene in the cells of said F_1 progeny fish.

It is an object of the invention to provide transgenic fish that may be used in, for example, an animal model of disease, preferably cancer. Preferably the cancer is a human cancer.

It is another object of the invention to provide transgenic fish that may be used as an animal model to discover drugs or genetic modifiers that sensitize

tumor cells over-expressing BCL2 or cMYC to the apoptosis-inducing effects of treatment with radiation or chemotherapeutic drugs.

It is another object of the invention to provide methods of making the above-referenced transgenic fish.

It is yet another object of the invention to provide methods of using the above-referenced transgenic fish, including using the fish in methods of screening for drugs or agents that modulate disease, particularly oncogene-mediated transformation, and for the discovery of drugs, agents and/or genes involved in the neoplastic or hyperplastic process, including tumor suppressor genes and potential drug targets.

It is a further object of the invention to provide methods of co-expressing more than one mammalian oncogene in a transgenic fish, including, for example, to determine accelerated onset of disease.

These and other objects and advantages of the present invention will be apparent from the descriptions herein.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Figures 1A-1H depict views of transgenic fish produced by introduction of nucleic acid comprising the *RAG2* promoter–mouse *cMYC* (*mcMYC*) transgene (also referred to herein as *RAG2-mcMYC*) and shows the external morphology of tumorigenic *RAG2-mcMYC* fish, as more fully described in Examples 1B and 1C. Wild type fish are seen in Figures 1A, 1B, 1G, 1I, 1K, 1M and 1O; *RAG2-mcMYC* F_0 mosaic fish are seen in Figures 1C-1F, 1H, 1J, 1L, 1N and 1P. Figures 1A, 1C, and 1E, are side views of the fish and Figures 1B, 1D, and 1F are the top views of the same fish shown in Figures 1A, 1C, and 1E, respectively. Figure 1G is a side view of a *RAG2-GFP* fish while 1H is a side view of *RAG2-mcMYC* F_0 fish injected on the *RAG2-GFP* background and obtained by GFP fluorescence. Tumors are denoted by arrows and labeled nose (N), eye (E), fin (F), probable thymic tumors (T).

Figures 1I-1P depict views of hematoxylin/eosin-stained transverse sections of transgenic fish produced by introduction of nucleic acid comprising *RAG2-cMYC*, as more fully described in Examples 1B and 1C. Massive infiltration of lymphoblasts in fish with tumors (Figure 1J) is seen when compared with sections from wild type-fish (Figure 1I). Figures 1K-1L, kidney; Figures 1M-1N, musculature; Figures 1O-1P, nasal region. Tumors are denoted by arrows and labeled fin (F), kidney (K); muscle (M); skin (S) and gut (G). Figures 1I, 1K, 1M, and 1O depict wild-type fish, and Figures 1J, 1L, 1N, and 1P represent transgenic fish with tumors. Scale bars in Figures 1I-J represent 1 mm, in Figures 1K-N represent 100 microns, and in Figures 1O-P represent 500 microns.

Figure 2. Figure 2 depicts views of transverse paraffin sections of transgenic fish produced by introduction of nucleic acid comprising *RAG2-cMYC* and analyzed for RNA expression of T-cell genes, as more fully described in Example 1C, in order to identify tumor cell lineage. Figures 2A-2B, RNA *in situ* analysis with a mouse *cMYC* RNA probe; Figures 2C-2D, zebrafish *RAG2*; and

Figures 2E-2F, zebrafish LCK. Antisense probes are used in Figures 2A, 2C and 2E and sense control probes are used in Figures 2B, 2D and 2F. All sections shown in Figures 2A-2F are of infiltrating T-cells in the body musculature.

Figures 2G-2H depict whole body transverse serial sections stained with hematoxylin/eosin (Figure 2G) or with anti-GFP antibody on RAG2-EGFP-mcMYC expressing F_0 mosaic fish (Figure 2H).

Figure 2I is a Southern analysis of tumor fish (T1-T3) and wild-type fish (WT1-WT4), as more fully described in Example 1D. Tumor DNA extracted from the tail was digested with Bgl-II and probed with the *TCRα-* and *IgM* constant region-specific probes. Bgl-II cuts within the constant region of *IgM*, resulting in two bands being detected (T2 and WT4). Four bands were detected in the remaining DNA samples and results from polymorphisms found within the *IgM* gene locus (T1, T3, and WT1-WT3).

Figure 3. Figures 3A-3F depict a fluorescence activated cell sorting (FACS) analysis and blast morphology of tumors formed in transgenic fish having nucleic acid comprising *RAG2-cMYC* as more fully described in Example 1C. Gated populations include red blood cells (red), lymphoid cells (blue), myeloid cells (green), and progenitors (pink). Sorted kidney blasts were reanalyzed by FACS (Figure 3C) and analyzed morphologically by May-Grunwald staining of cytospin cell preparations as shown in Figure 3F, which depicts a representative field of sorted blasts. Populations of cells within each gate are noted as percent of total cells. Forward scatter (FSC-H) and side scatter (SSC-H). Images in 3F were taken at 1000x magnification.

Figure 4. Figure 4A depicts a top view of a wild-type AB fish that received transplanted tumor cells as a 2 day-old embryo at 44 days, as more fully described in Example 1E.

Figures 4B-4D depict touch preps of body musculature of transgenic fish. Figure 4B depicts a touch prep of F_0 mosaic *RAG2-mcMYC* tumor-bearing fish;

Figure 4C depicts transplanted tumor cell fish from Figure 4A; and Figure 4D depicts wild-type fish. Scale bars in Figure 4B-4D represent 10 microns.

Figure 4E depicts an agarose gel stained with ethidium bromide showing polymerase chain reaction (PCR) analysis of genomic DNA isolated from the tail musculature of wild-type AB fish (WT), F_0 mosaic fish (F_0-mosaic), and embryo with tumor cell transplant from Figure 4A (Transplant). PCR was completed on the plasmid used for injection as a positive control (+ control). The molecular weight marker (MWM) and band sizes are noted.

Figures 4F-4I depict hematoxylin/eosin stained sections of the kidney (Figures 4F-G) and musculature (Figures 4H-I). Adult wild-type fish were sublethally irradiated with 2500 Rads and injected with whole kidney marrow. Figure 4F-H are irradiated controls and Figures 4G-I are transplants. Scale bars in Figures 4F-I represent 50 microns.

Figure 4J depicts a flow cytometric analysis of a tumor from *RAG2-mcMYC* F_0 mosaic fish to determine DNA content as more fully described in Example 1.

Figure 5. Figure 5 depicts an example of a plasmid vector that may be used to incorporate an oncogene sequence to produce an oncogene protein fused to a green fluorescent protein (GFP). GM2 denotes the green fluorescent open reading frame and pA denotes the synthetic polyadenylation sequence used in the construct. T7 and T3 denote promoters used for *in vitro* transcription and vector sequence is noted as single lines on the circle. Restrictions sites are noted. For oncogene constructs, the *GM2* open reading frame has been excised and replaced with sequences for mouse *cMYC, EGFP-mcMYC, EGFP-BCL2, TAN1, and HOX11.*

Figure 6. Figure 6 depicts one example of a potential ethyl-nitrosurea (ENU)-induced genetic modifier screen in the zebrafish. Zebrafish lines are indicated as X plus superscript of either wik or AB. Point mutations induced by

ENU are noted as asterisks and are linked to the *wik* alleles. Transgenic lines are noted (*RAG2-GFP* as EGFP and *RAG2-mcMYC* as cMYC) and plus signs designate no transgene being passed on (*i.e.,* the fish are heterozygotes if they are cMYC/+ for example, but homozygous for the transgene if they are cMYC/cMYC). Breeding is designated by converging arrows. Mutations will be identified in the generation noted by "Modifier Mutation" while the line is maintained without disease in the generation noted by "Line Maintained."

Figure 7. Figure 7 depicts the *RAG2-Lox-dsRED2-EGFP-mMYC* construct. A) A diagram of the construct. B) Transient injection of this construct drives expression of dsRED in developing mosaic fish while the *EGFP-mMYC* transgene is not expressed, as determined by GFP fluorescent microscopy (C). Poly-adenylation and transcription stop sites note (pA).

Figure 8. Figure 8 depicts CRE-mediated excision of *dsRED2* allele in fish transiently injected with both the *CMV-Lox-dsRED2-EGFP* and *PCS2+CRE* plasmids. A) A diagram of the construct. B) Transient injection of the *CMV-Lox-dsRED2-EGFP* construct in the absence of *PCS2+CRE* plasmid drives expression of dsRED in developing mosaic fish (B) but not GFP (C). Meanwhile, fish injected with both the *CMV-Lox-dsRED2-EGFP* and *PCS2+CRE* plasmids are both sdRED2 (D) and GFP labeled (E). Poly-adenylation and transcription stop sites note (pA).

Figure 9. Figure 9 depicts CRE-mediated excision of *dsRED2* allele in fish transiently injected with both the *CMV-Lox-dsRED2-EGFP* and *CRE* RNA. Bright field images of injected fish (A,D). Transient injection of the *CMV-Lox-dsRED2-EGFP* construct in the absence of *CRE* RNA drives expression of dsRED in developing mosaic fish (B) but not GFP (C). Meanwhile, fish injected with both the *CMV-Lox-dsRED2-EGFP* and *CRE* RNA fail to express sdRED2 (E) but strongly express GFP (F). This excision event is 100% efficient.

Figure 10. Figure 10 depicts RT-PCR expression of T-ALL oncogenes in zebrafish MYC-induced leukemias. Tumor samples are noted by numbers. T-ALL oncogenes include *SCL, LMO2, LMO1, HOX-11, TLX-3a*, and *TLX-3b. Beta-actin* and *EF1-alpha* are housekeeping genes and verify that equal amounts of cDNA were used in each sample.

Figure 11. Figure 11 depicts RNA *in situ* hybridization of paraffin embedded sections with Scl and Lmo2. Anti-sense probes (a-) and control sense probes (s-). MYC-G and MCMYC are tumors from mosaic injected *RAG2-mMYC* fish.

Figure 12. Figure 12 depicts the amino acid alignment and phylogenetic analysis of vertebrate BCL2 proteins. (A) Alignment of BCL2 and BCL-xL proteins from zebrafish (z), Xenopus (x), chicken (c), and human (h). Amino acid residues conserved among both BCL2 and BCL-xL family members are indicated (#) while amino acid residues conserved among only BCL2 proteins are noted (*). Conserved BH1, BH2, BH3, and BH4 domains are denoted by a single line above the alignment. Dashes denote gaps introduced to maximize alignment. Sequence alignments were made using Megalign. (B) Diagram showing conserved domain homologies when zebrafish bcl-2 is compared to the human BCL2 protein. (C) Phylogenetic analysis of vertebrate BCL2 and BCL-xL proteins.

Figure 13. Expression of the EGFP-zBCL2 fusion protein during embryogenesis rescues developmentally regulated apoptosis. (A) Diagram of the *EGFP-zBCL2* fusion transgene. TUNEL stained 16 hpf embryo injected with GFP control RNA (B) or *EGFP-zBCL2* RNA (C). Graph showing that number of apoptotic cells in the embryo is significantly decreased in embryos injected with *EGFP-zBCL2* RNA when compared to uninjected control or GFP injected control fish. $P<.0001$.

Figure 14. Expression of the EGFP-zBCL2 fusion protein during embryogenesis rescues radiation-induced apoptosis. Tunel stained 20 hpf embryo injected with *GFP* control RNA (A) or *EGFP-zBCL2* RNA (B) following irradiation at 14 hpf.

Figure 15. T-cells from 6-day-old *RAG2-EGFP-zBCL2* transgenic fish are resistant to irradiation-induced apoptosis. Eight-day-old *RAG2-GFP* (GM2) without irradiation (A) or two days post-irradiation treatment (B). Eight-day-old *RAG2-EGFP-zBCL2* transgenic fish without irradiation (C) or two days post-irradiation treatment (D). Asterisk denotes auto-fluorescence of the swim bladder. Fish oriented with anterior to the left and dorsal to the top.

Figure 16. T-cells from 3-month-old *RAG2-EGFP-zBCL2* transgenic fish are resistant to irradiation-induced apoptosis. *RAG2-GFP* without irradiation (A) or four days post-irradiation treatment (B). *RAG2-EGFP-zBCL2* transgenic fish without irradiation (C) or four days post-irradiation treatment (D). Fish oriented with anterior to the left and dorsal to the top.

Figure 17. T-cells from 5 day old *RAG2-GFP* fish are ablated by dexamethasone treatment by 8 dpf. *RAG2-GFP* control fish treated with 0.1% ethanol (A). *RAG2-GFP* fish treated with 250 milligrams/microliter (B) or 25 milligrams/microliter of dexamethasone (C,D). All 6 fish treated with 250 milligrams/microliter of dexamethasone lacked GFP-labeled T-cells by 8 dpf (3 days of treatment) while 2 of 6 fish treated with 25 milligrams/microliter of dexamethasone lacked GFP-labeled cells (D). Some fish in the 25 milligrams/microliter treatment group had significantly less T-cells than when compared to controls (C).

Figure 18. Dominant genetic screen to identify suppressors of BCL2 function in *RAG2-EGFP-zBCL2* fish. ENU induced point mutations in X gene are noted by asterisk and are linked to the Wik allele (X^{Wik*}) or wild-type X alleles in

the AB strain are noted (X^{AB}). Five-day-old fish will be irradiated (15 Gy) and analyzed for loss of GFP-positive T-cells by 8dpf.

Figure 19. Recessive genetic screen to identify suppressors of BCL2 function in *RAG2-EGFP-zBCL2* fish. ENU induced point mutations in X gene are noted by asterisk and are linked to the *Wik* allele (X^{Wik*}) or wild-type X alleles in the AB strain are noted (X^{AB}). Five day old fish will be irradiated (15 Gy) and analyzed for loss of GFP-positive T-cells by 8dpf.

Figure 20. Recessive genetic screen to identify suppressors or enhancers of *MYC*-induced leukemia. Adult zebrafish males are treated with ENU and mated to transgenic female fish which contain a conditional *MYC* allele targeted to T-cells by the *RAG2* promoter. The eggs from the F_1 females obtained by squeezing and fertilized *in vitro* by UV-inactivated sperm and subject to early pressure. The resulting F_2 gynogenetic diploid embryos will the be induced to express the conditional *EGFP-mMYC* transgene, generating tumor prone fish. The larvae will be analyzed for variation in the onset of T-ALL.

DETAILED DESCRIPTION OF THE INVENTION

For the purposes of promoting an understanding of the principles of the invention, reference will now be made to preferred embodiments, and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the invention is thereby intended, such alterations and further modifications of the invention, and such further applications of the principles of the invention as illustrated herein, being contemplated as would normally occur to one skilled in the art to which the invention relates.

The present invention relates to transgenic fish and methods for their production and use. In one aspect of the invention, transgenic fish are provided whose genome has stably-incorporated therein nucleic acid comprising an oncogene operably linked to a promoter. In yet other aspects of the invention, methods of making and methods of using the transgenic fish are provided. In certain forms of the invention, a method of screening for, or otherwise identifying, drugs or agents that modulate oncogene-mediated neoplastic or hyperplastic transformation or increase the sensitivity of transgenic cells to the toxic effects of radiation or chemotherapy are provided. Additionally, the transgenic fish described herein may be used as a model to study conserved pathways that lead to oncogene-mediated cancer progression in vertebrates, including, for example, non-Hodgkin's lymphoma, high-grade astrocytoma, rhabdomyosarcoma, neuroblastoma, neuorendocrine carcinoma, pancreatic carcinoma, ovarian carcinoma, testicular carcinoma, stomach cancer, colon cancer, renal cancer melanoma and acute or chronic myeloid leukemia, and *cMYC*-induced T-cell acute lymphoblastic leukemia.

In one aspect of the invention, stably transformed transgenic fish are provided. In one embodiment, a transgenic fish has a genome which has stably-integrated, or otherwise incorporated, therein an introduced oncogene operably linked to a promoter. The promoter is preferably an organ- or tissue-specific

(including cell-specific) promoter or a promoter that can be regulated in a specific tissue. Most preferably, the promoter is a lymphoid-specific promoter, including T-cell or B-cell-specific promoters, such as a *RAG1* or *RAG2* promoter. The oncogene is typically from an animal other than a fish or from the fish itself and may advantageously be part of a recombinant vector as further described herein. Preferably the oncogene is a mammalian oncogene. Such fish may form a stable fish line in that they have the capacity to reproduce and pass their genetic information relating to the oncogene to their progeny.

A wide variety of fish may be utilized in the invention. Exemplary fish include teleost fish, such as zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), mummichog (*Fundulus heteroclitus*), killifish (Genus *Fundulus*), catfish (Genus *Ictalurus*), such as channel catfish; carp (Genus *Cyprinus*), such as common carp; and trout or salmon (*e.g.*, Genus *Salvelinus*, *Salmo*, and *Oncorhynchus*).

Zebrafish, in particular, may be advantageously utilized as compared to other animal models. For example, zebrafish are amenable to genetic screens, modifier screens, and chemical screens; develop rapidly *ex-utero*; are transparent for much of their life cycle and produce large clutches of offspring weekly. Zebrafish can be raised in relatively small facilities (housing up to about 54 adult fish in a single 9 liter tank), and can reliably produce offspring in large quantities, with each mature female typically laying between 100 to 300 eggs per week. These eggs are fertilized externally, and the embryos are transparent allowing the early development of hematopoietic tissues and other organ and tissue systems to be directly observed using only a dissecting microscope. Embryonic development is extremely rapid with most organ systems including blood cell formation being fully developed by 5 days post fertilization. Full reproductive maturation is reached by about 3 months.

The vector includes an oncogene operably linked to a promoter. Preferably the promoter is an organ- or tissue-specific promoter. As known in the art, an oncogene is a gene whose expression can lead to alteration of the control of

cellular proliferation or to the prevention of programmed cell death. A wide variety of oncogenes may be utilized in the nucleic acid constructs described herein. The oncogenes may be of viral or cellular origin. Oncogenes of cellular origin include endogenous oncogenes. Such oncogenes, when expressed, lead to neoplastic or hyperplastic transformation of a cell. Exemplary oncogenes include *MYC, SRC, FOS, JUN, MYB, RAS, ABL, BCL2, HOX11, HOX11L2, TAL1/SCL, LMO1, LMO2, EGFR, MYCN, MDM2, CDK4, GLI1, IGF2,* activated *RAS*, activated *EGFR*, mutated genes, such as *FLT3-ITD,* mutated and activated versions of *TP53, PAX3, PAX7, BCR/ABL, HER2/NEU, FLT3R, FLT3-ITD,SRC, RAS, ABL, TAN1, PTC, B-RAF, PML-RARα, E2A-PBX1,* and *NPM-ALK,* as well as fusion of members of the *PAX and FKHR* gene families.

Other exemplary oncogenes are well known in the art and several such examples are described in, for example, *The Genetic Basis of Human Cancer* (Vogelstein, B. and Kinzler, K.W. eds.. McGraw-Hill, New York, NY, 1998), such as in Tables 5.1-5.3 of Look, A.T., *Genes altered by chromosomal translocations in leukemias and lymphoma*, pages 109-141, which is incorporated herein by reference in its entirety. Mammalian homologues of such genes are preferred because they can be distinguished from endogenous fish genes. Further preferred are human homologues of such genes. The corresponding sequences of such oncogenes, including the human homologues of the oncogenes, are known and may be found, for example, in the NCBI database (www.ncbi.nlm.nih.gov).

The oncogene is selected based on the form of cancer it is desired that the transgenic fish will develop. For example, mutated or activated genes of the *RAS* family may be used for induction of a wide variety of types of cancers, such as renal, pancreatic or colon cancers, and *HOX11* and *TAL1* may be used for T-cell cancer induction, *etc.* Preferably the oncogenes are T-cell or B-cell oncogenes. Most preferably, the T-cell oncogenes are members of the *MYC*, T*AL1/SCL, TAL2, LYL1, LMO1, LMO2, HOX11, HOX11L2, TAN1,* and *LYL1* gene families, and the B-cell oncogenes are members of the *MYC, E2A-PBX1,*

E2A-HLF, TEL-AML1, BCL6, BCL3, LYT10, MLL, HOX or *PAX5* gene families. In one form, the oncogene is *MYC*, such as *cMYC* (GenBank Accession No. XM_122917.1, available at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide). Expression of such a nucleotide sequence in T-cell progenitors of the fish leads to development of acute T-cell lymphoblastic leukemia or lymphoma. The invention is not limited to such an oncogene sequence. For example, altered forms of the oncogene nucleotide sequence, such as *cMYC* or the other oncogene nucleotide sequences described herein, that increase or decrease the transformation potential of the oncogene are also envisioned.

In one form of the invention, the oncogene utilized in the invention may have a *cMYC* nucleotide sequence (GenBank Accession No. XM_122917.1) that has at least about 60%, preferably at least about 70%, more preferably at least about 80%, and most preferably at least about 90% identity to the nucleotide sequence or the other oncogene nucleotide sequences discussed herein.

Percent identity may be determined, for example, by comparing sequence information using the advanced BLAST computer program, version 2.0.8, available from the National Institutes of Health (www.ncbi.nlm.nih.gov/BLAST). The BLAST program is based on the alignment method of Karlin and Altschul, (1990) *Proc. Natl. Acad. Sci. USA* 87:2264-2268 and as discussed in Altschul, *et al.*, (1990) *J. Mol. Biol.* 215:403-410; Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* (1993) 90:5873-5877; and Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389-3402.

Additionally, the oncogene may include nucleotide sequences having substantial similarity to the *cMYC* nucleotide sequence (GenBank Accession No. XM_122917.1) or the other oncogene nucleotide sequences discussed herein. By "substantial similarity", it is meant herein that the nucleotide sequence is sufficiently similar to a reference nucleotide sequence that it will hybridize therewith under moderately stringent conditions. This method of determining

similarity is well known in the art to which the invention pertains. Briefly, moderately stringent conditions are defined in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed. Vol. 1, pp. 101-104, Cold Spring Harbor Laboratory Press (1989) as including the use of a prewashing solution of 5X SSC (a sodium chloride/sodium citrate solution), 0.5% sodium dodecyl sulfate (SDS), 1.0 mM ethylene diaminetetracetic acid (EDTA) (pH 8.0) and hybridization and washing conditions of 55°C, 5X SSC. A further requirement of the nucleotide sequence of the oncogene is that it encode a protein having cell neoplastic transformation ability. That is, the proteins have the ability to convert normal (*i.e.*, non-cancerous cells) into cancerous cells (*i.e.*, tumors).

The oncogene, also considered herein as the transgene, the gene which is introduced into the genome described herein, may be either synthesized *in vitro* or isolated from a biological source. Such methods of synthesis and isolation are well known to the skilled artisan.

The oncogene is operably linked to a promoter. Preferably the promoter is a organ- or tissue- (including cell-) specific promoter. Most preferably, the promoter is a lymphoid-specific promoter. For example, by "lymphoid-specific," it is meant herein that the promoter drives expression of the oncogene only in tissue of lymphoid origin, such as in B- and T-cell progenitor cells known to the art. The lymphoid-specific promoters may be derived from any lymphoid-specific genes. Exemplary lymphoid-specific promoters that may advantageously be used in the invention include promoters of the recombination activating genes (RAG), including *RAG1* and *RAG2*; *LCK*, which encodes a T-cell-specific, non-receptor tyrosine kinase; IgM enhancer elements, and *CD2.* Several promoters that direct tissue-restricted expression have been identified, for example, zebrafish *RAG1* (Jessen *et al.*, *Nat. Genet., 23*: 16-17 (1999)) and zebrafish *RAG2* (Jessen *et al.*, *Genesis*, *29*: 156-162, (2001)) for lymphoid tissues, *Keratin-8* for epithelial cells (Gong *et al.,* 2002), *Islet-1* for neural-specific expression (Motoike *et al.*, *Genesis*, 28:75-81 (2000)), *PDX-1* and *Insulin* for pancreas (Huang *et al.*, *Genesis*, 30:134-6 (2001)), *GFAP* for glial cells, and

MYO-D and *alpha-actin* for muscle (Higashima, 1997). Promoters having at least about 70% identity, at least about 80% identity, and further at least about 90% identity to the nucleotide sequences of the tissue-specific promoters described herein are also envisioned, provided that they promote transcription of the oncogene to which they are operably linked. Since most mammalian promoters are found not to work well in fish, then the genomic regulatory sequences of the zebrafish, fugu or other fish species often must be specifically cloned upstream, within, and downstream of the coding sequence of interest, which may be accomplished by procedures routine to those skilled in the art. In certain embodiments, the promoter is T-cell progenitor-specific in that it will drive expression of the oncogene only in T-cell progenitors. Such a construct may be made by using the *RAG2* or *LCK* genomic sequences upstream of the coding region of the gene. The genomic sequences are first cloned upstream of *GFP* to see if they can drive expression of this fluorescent marker in a tissue-specific fashion during development, and if so, then the same sequences are used to drive the expression of *cMYC* and other oncogenes. Similar procedures may be utilized for construction of other, *e.g.,* zebrafish, organ- and tissue-specific promoters, which are well known to those of skill in the art, such as those cloned from the genes encoding tyrosine hydroxylase for the dopaminanergic nervous system, *MYO-D* for the muscle system, and *MPO* or *PU.1* for the myeloid system.

As defined herein, a nucleotide sequence is "operably linked" to another nucleotide sequence when it is placed in a functional relationship with another nucleotide sequence. For example, if a coding sequence is operably linked to a promoter sequence, this generally means that the promoter may promote transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary join two protein coding regions, contiguous and in reading frame. Since enhancers may function when separated from the promoter by several kilobases and intron sequences may be of variable lengths, some nucleotide sequences may be operably linked but not contiguous.

The transgene may be included in a vector for delivery. A vector, as used herein and as known in the art, refers to a nucleic acid construct that includes genetic material designed to direct transformation (*i.e.*, the process whereby genetic material of an individual cell is altered by incorporation of exogenous DNA into its genome) of a targeted cell. A vector may contain multiple genetic elements positionally and sequentially oriented, *i.e.*, operably linked with other necessary or desired elements such that the nucleic acid in a cassette can be transcribed and, if desired, translated in the microinjected, single-cell fertilized embryo.

Recombinant expression vectors may be constructed by incorporating the above-recited nucleotide sequences within a vector according to methods well known to the skilled artisan and as described, for example, in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, 2nd ed., Cold Springs Harbor, New York (1989). Other references describing molecular biology and recombinant DNA techniques include, for example, *DNA Cloning 1: Core Techniques*, (D. N. Glover, *et al.*, eds., IRL Press, 1995); *DNA Cloning 2: Expression Systems*, (B. D. Hames, *et al.*, eds., IRL Press, 1995); *DNA Cloning 3: A Practical Approach*, (D. N. Glover, *et al.*, eds., IRL Press, 1995); *DNA Cloning 4: Mammalian Systems*, (D. N. Glover, *et al.*, eds., IRL Press, 1995); *Oligonucleotide Synthesis* (M. J. Gait, ed., IRL Press,1992); *Nucleic Acid Hybridization: A Practical Approach,* (S. J. Higgins and B. D. Hames, eds., IRL Press, 1991); *Transcription and Translation: A Practical Approach,* (S. J. Higgins & B. D. Hames, eds., IRL Press, 1996); R. I. Freshney, *Culture of Animal Cells: A Manual of Basic Technique*, 4th Edition (Wiley-Liss, 1986); and B. Perbal, *A Practical Guide To Molecular Cloning, 2nd Edition,* (John Wiley & Sons,1988); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., John Wiley & Sons), which is regularly and periodically updated.

A wide variety of vectors are known that have use in the invention. Suitable vectors include plasmid vectors, viral vectors, including retrovirus vectors (*e.g.*, see Miller *et al.*, *Methods of Enzymology*, 217:581-599 (1993)),

adenovirus vectors (*e.g.*, see Erzurum, *et al. Nucleic Acids Res.*, 21:1607-1612 (1993); Zabner, *et al.*, *Nature Genetics*, 6:75-83 (1994); and Davidson, *et al.*, *Nature Genetics*, 3:219-223 (1993)) adeno-associated virus vectors (*e.g.*, see Flotte, *et al.*, *PNAS* 90:10613-10617 (1993)), herpesvirus vectors (*e.g.*, see Anderson, *et al.*, *Cell Mol. Neurobiol.*, 13:503-515 (1993)), and lentivirus vectors (*e.g.,* see Lever, *Curr. Opin. Mol. Ther.*, 2:488-496 (2000)). The vectors may include other known genetic elements necessary or desirable for efficient expression of the nucleic acid in a specified host cell, such as the transgenic fish host cells described herein, including regulatory elements. For example, the vectors may include a promoter, including one that is specific to organ- or tissue-specific (*e.g.,* specific to lymphoid tissue*)* as described herein and any necessary enhancer sequences that cooperate with the promoter to achieve transcription of the gene. By "enhancer" is meant nucleotide sequence elements which can stimulate promoter activity in a cell, such as a transgenic fish host cell described herein. The vectors may be in, for example, a linearized form.

The oncogene nucleotide sequence may also be fused to a nucleotide sequence encoding a reporter gene product so that a fusion protein will be formed, and whose presence and or location may be visualized or otherwise identified. The terms "encoding" and "coding" refer to the process by which a nucleotide sequence, through the mechanisms of transcription and translation, provides the information to a cell from which a series of amino acids can be assembled into a specific amino acid sequence to produce a polypeptide. As one example of such a nucleotide sequence, a nucleotide sequence encoding GFP may be advantageously utilized in the invention so that areas of the developing embryo and/or hatched or otherwise mature fish will fluoresce upon expression of the fusion protein. Alternatively, other reporter gene products may be utilized, including luciferase, β-galactosidase, chloramphenicol acytransferase, β-glucuronidase and alkaline phosphatase. Assays for determining the presence, and including determining the activity or amount, of the reporter gene products described herein are known to the art and are

discussed in, for example, *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., John Wiley & Sons), which is regularly and periodically updated. Further descriptions of assays for the reporter gene products discussed herein may be found, for example, in the following publications: for luciferase, see Nguyen, V.T. *et al.*, *Anal. Biochem.* 171:404-408 (1988); for β-galactosidase, see, *e.g.*, Martin, C.S., *et al.*, in *Bioluminescence and Chemiluminescence: Molecular Reporting with Photons* pp. 525-528 (J.W. Hastings, *et al.*, eds., John Wiley & Sons,1997); Jain, V.K. and Magrath, I.T., *Anal. Biochem.* 199:119-124 (1991); for β-galactosidase, β-glucuronidase and alkaline phosphatase see, for example, Bronstein, I. *et al.*, in *Biolumunescence and Chemiluminescence: Fundamentals and Applied Aspects*, pp. 20-23, (A.K. Campbell, *et al.*, eds., John Wiley & Sons, 1994); for chloramphenical acetyltransferase, see Cullen, B., *Methods. Enzymol.* 152:684 (1987); Gorman, C. *et al. Mol. Cell. Biol.* 2:1044 (1982); Miner, J.N. *et al.*, *J. Virol.* 62:297-304 (1988);Sleigh, M.J., *Anal. Biochem* 156:251-256 (1986); Hruby, D.E. and Wilson, E.M., *Methods Enzymol.* 216:369-376 (1992).

An example of a suitable plasmid vector including a murine cMYC-encoding nucleotide sequence and a GFP-encoding nucleotide sequence (Yi *et al.*, 2001) regulated by the zebrafish *RAG2* promoter that may be used in the invention to produce an oncogene-green fluorescent fusion protein is shown in Figure 5. This fusion construct induced lymphoma in zebrafish in a similar manner as cMYC alone. Other oncogenes that may be directed to specific organs or tissues, such as lymphoid tissues, by specific promoters and fused to GFP include members of the activated-*RAS, BCL2, HOX11, HOX11L2, LMO1, LMO2,* and *TAL1/SCL* gene families. As described herein, other reporter genes well known to those of skill in the art may also be used for oncogene fusions.

In yet another aspect of the invention, the oncogene is preceded by a reporter gene, such as a fluorescent protein gene (*e.g.,* GFP, RFP, BFP, YFP, or dsRED2) or a luciferase protein gene, comprising a strong transcriptional stop-site, which is flanked by site specific recombinase recognition sites (*e.g.,* Flox, Lox, or FRT-sites). A ubiquitous gene promoter (*e.g., EF1-alpha* or *beta-actin*)

may drive expression of the "Loxed," "Floxed" or "FRPed" reporter gene. A second gene product (*e.g.,* an oncogene) is adjacent to the reporter gene but is not expressed in the absence of recombinase protein expression because of the strong transcription stop-site within reporter gene. However, when the recombinase protein expression is activated in the cells, the Loxed, Floxed, or FRPed reporter gene product is excised, and the second gene is juxtaposed to the ubiquitous gene promoter. Additionally, tissue-specific recombination may be facilitated by laser-activation of a heat-shock inducible site-specific recombinase transgene through use of a laser. Laser activation may be targeted to individual cells during embryologic development. This transgenic strategy not only prevents toxicity to the parental cells harboring the oncogene, but also allows the use of two transgenic fish lines to create a wide variety of cancer models, which express the same oncogene. For example, the *MYC* oncogene is misregulated in T-cell leukemia/lymphoma, Burkitt's and other non-Hodgkin's lymphoma, pancreatic beta cell tumors, invasive islet adenocarcinoma, renal cell carcinoma, ovarian cancer, Acute Myeloid Leukemia, colon carcinoma, glioblastomas, and melanoma. Thus, all of these diseases may be modeled using the strategy described herein.

In yet another aspect of the invention, methods of making a transgenic fish are provided herein. In one embodiment, a method includes introducing into a fertilized fish egg (*i.e.*, including a fish embryo) or an unfertilized fish egg nucleic acid including a mammalian oncogene operably linked to a promoter. The nucleic acid may be part of a vector described herein. When a fertilized fish egg is used, the method includes developing the fish embryo into a transgenic fish. When the oncogene is introduced into a non-fertilized egg, the method includes fertilizing the egg and developing the fish embryo into a transgenic fish. The nucleic acid construct may be introduced into the egg by a variety of methods known to the art, including mechanical methods, chemical methods, lipophilic methods, retroviral infection methods, and electroporation. Exemplary mechanical methods include, for example, microinjection. Exemplary chemical methods include, for example, use of calcium phosphate or DEAE-Dextran.

Exemplary lipophilic methods include use of liposomes and other cationic agents for lipid-mediated transfection. Such methods are generally well known to the art and many of such methods are described in, for example, *Gene Transfer Methods: Introducing DNA into Living Cells and Organisms*, (P.A. Norton and L.F. Steel, eds., Biotechniques Press, 2000); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., John Wiley & Sons), which is regularly and periodically updated. Microinjection techniques involving fish are further more fully described in, for example, Chen, T.T. and Powers, D.A., *Trends. Biotechnol.* 8:209-215 (1990) and Fletcher, G.L., and Davis, P.L., *Transgenic fish for aquaculture*, in *Genetic Engineering* (Setlow, J.K., ed., Plenum Press, 1991). Electroporation techniques involving fish are further more fully described in, for example, Powers, D.A., *et al.*, *Molec. Mar. Biol. Biotechnol.* 1:301-308 (1992) and Lu, J.K., *et al.*, *Molec. Mar. Biol. Biotechnol.* 1:366-375 (1992). Techniques for introducing DNA into fish eggs or embryos by infection with retroviral vectors, such as pantropic retroviral vectors, are further described in, for example, Burns, J.C., *et al.*, *Proc. Natl. Acad. Sci. USA* 90:8033-8037 (1993).

The vector or other nucleic acid comprising the transgene may be introduced into an unfertilized egg or a fertilized egg at a desired stage of development. Multiple vectors, each encoding different transgenes as described herein may be used. When using a fertilized egg, or embryo, it is preferred to introduce the nucleic acid into the embryo (*i.e.*, at the one-cell stage of development). However, the nucleic acid may also be administered at later stages of development, including the two-cell stage, four-cell stage, *etc.* Therefore, the nucleic acid may be introduced into the morula, blastula, *etc.* At least one isolated nucleic acid molecule incorporating the above-described transgenic construct is introduced into the zygote. Additionally, when the nucleic acid is introduced into an egg at later stages of development, at least one isolated nucleic acid molecule incorporating the above-described transgenic construct is introduced into at least one cell of the, for example, morula, blastula, *etc.*

Fish eggs may be obtained from the appropriate fish by standard methods. Many of the fish may be purchased commercially from, for example, pet stores. Fertilized eggs may be obtained by methods known to the art. For example, a desired number of appropriately aged fish, such as about three to about twelve month old fish, with a desired ratio of females to males (such as about 2:1) may be placed in an appropriately sized container, such as a tank. Eggs may be collected by, for example, placing the fish in a nuptial chamber in the tank for an appropriate time after mating, such as about 10 to 60 minutes. Such methods are described in, for example, Culp, P. *et al., Proc. Natl. Acad. Sci.* 88:7953-7957 (1991). Alternatively, fish eggs may be artificially fertilized by methods known to the skilled artisan. One skilled in the art is familiar with other methods of obtaining such fertilized fish eggs.

After introducing the nucleic acid construct into the fish egg or embryo, the fish egg or embryo is provided with an environment conducive to development into an adult fish. Such an environment may include, for example, growth at 28.5°C in E3 egg water for 15 days followed by introduction into circulating system water by day 16 (*The Zebrafish Book: A Guide For the Laboratory Use of Xebrafish (Danio rerio),* 4th Ed. (M. Westerfield *ed.*, University of Oregon Press, Eugene, OR (2000)).

Transgenic fish produced as described herein may be identified by common procedures known to the art, including dot blot and Southern blot hybridization of genomic DNA. Briefly, such methods involve isolation of genomic DNA from tissues of the fish, digestion of DNA with restriction enzymes and Southern blot hybridization of the digested DNA products as described in, for example, Chen, T.T. *et al.*, *Biotech. Ann. Rev.* 2:205-236 (1996). A preliminary screen may be accomplished by isolating genomic DNA from a piece of fin tissue, amplifying the transgenic sequence by the polymerase chain reaction and Southern blot analysis of the amplified products as described in Lu, J.K. *et al.*, *Molec. Mar. Biol. Biotechnol* 1:366-375 (1992) and Chen, T.T. *et al.*, Molec. Mar. Biol. Biotechnol. 2:88-95 (1993). Additionally, if an oncogene-fluorescent fusion

protein, including an oncogene-GFP fusion protein, is encoded by the introduced nucleic acid, a visual preliminary screen for fluorescence may be used.

The transgenic fish produced preferably has the transgene stably integrated into its genome. This means that the transgene is integrated into the genome of the fish as opposed to being extrachromosomal. It is additionally preferred that the transgene is found in germ cells and in B- and/or T-cell progenitors or other organ- or tissue-specific cells of the fish. The presence of the transgene in the germ cells allows for transmission of the transgene to subsequent generations. In certain embodiments, it is preferred that the transgene is expressed in the B- or T-cell progenitors so the fish will develop a lymphoma or leukemia. In one embodiment, the transgene is expressed in T-cell progenitors so that fish develop a T-cell lymphoblastic leukemia, such as T-cell acute lymphoblastic leukemia. In other embodiments, the fish develop B-cell lymphoma and leukemias, myeloid leukemias, nervous system tumors (*e.g.,* brain tumors), melanoma, bowel tumors, pancreatic tumors, skin tumors, muscle sarcomas, germ cell tumors, ovarian carcinomas, or other tumors or cancers well known by those of skill in the art. Such an animal may thus advantageously be used as a model for cancer, including, for example, as a model for T-cell acute lymphoblastic leukemia. Accordingly, methods of screening for drugs or agents for modulating oncogene-mediated neoplastic or hyperplastic transformation, as well as the sensitivity of transgenic cells to treatments with radiation or chemotherapy, are provided.

In another aspect of the invention, methods of making a stable transgenic fish model of human cancers are provided, wherein an oncogene can be regulated in any tissue by using regulatable site-specific recombinases. In one embodiment, a method includes: (a) producing a first transgenic fish whose genome has stably-integrated therein a first transgene cassette comprising a Floxed, Loxed, or FRPed reporter gene (*e.g.,* a fluorescent protein gene or luciferase), comprising a strong transcription stop-site, wherein said reporter gene is regulated by a ubiquitous gene promoter (*e.g., beta-actin* or *EF1-alpha*),

and an oncogene placed immediately after the reporter gene; (b) producing a second transgenic fish whose genome has stably-integrated therein a second transgene cassette comprising a *flip* or *cre-recombinase* (*CRE*) gene operably linked to a heat shock inducible promoter (*e.g., HSP-70*); (c) mating said first transgenic fish with said second transgenic fish to produce offspring, which co-express said reporter gene and said *flip* or *CRE* gene; (d) laser-activating the heat-shock inducible *CRE* transgene in single cells of the body causing recombination of the first transgenic cassette, wherein the reporter gene is excised and the ubiquitous gene promoter is juxtaposed adjacent to the oncogene.

In another aspect, a method of screening for drugs or agents that modulate oncogene-mediated, or otherwise induced, neoplastic or hyperplastic transformation, or that modulate the sensitivity of transgenic cells to treatments with radiation or chemotherapy, is provided. A method comprises (a) contacting or otherwise exposing a transgenic fish (*e.g.,* an adult transgenic fish or a transgenic fish embryo) described herein with a test drug or agent, wherein the genome of the transgenic fish has stably integrated therein nucleic acid comprising an oncogene operably linked to a promoter; (b) determining if the test drug or agent suppresses or enhances oncogene-mediated neoplastic or hyperplastic transformation, or modulates the sensitivity of transgenic cells to treatments with radiation or chemotherapy; and (c) classifying the test drug or agent as a drug or agent that modulates oncogene-mediated neoplastic or hyperplastic transformation, or that modulates the sensitivity of transgenic cells to treatments with radiation or chemotherapy, if the test drug or agent suppresses or enhances oncogene-mediated neoplastic or hyperplastic transformation or modulates the sensitivity of transgenic cells to treatments with radiation or chemotherapy. As mentioned herein, the modulation may include suppressing, or otherwise decreasing, or enhancing, or otherwise stimulating, oncogene-mediated neoplastic or hyperplastic transformation, including the rate of oncogene-mediated neoplastic or hyperplastic transformation, or sensitivity of transgenic cells to treatments with radiation or chemotherapy.

The test drug or agent is typically identified from a large-scale, robotically-driven screen of thousands of compounds to identify a drug or agent thought to have the ability to modulate oncogene-mediated neoplastic or hyperplastic transformation, or modulate the sensitivity of transgenic cells to treatments with radiation or chemotherapy. Such screens are routine, and these, and other screening methods, are well known by those of skill in the art. The test drug or agent may suppress, or otherwise alter, or enhance expression of oncogene RNA and/or the oncogenic protein product, or RNA or protein expression of other genes involved in the oncogenic transformation process. Additionally, the test drug or agent may inhibit or stimulate the activity of other molecules involved, directly or indirectly, in the neoplastic/hyperplastic transformation process, or in the sensitivity of transgenic cells to treatments with radiation or chemotherapy. A wide variety of drugs or agents may be tested in the screening methods of the present invention. For example, small molecule compounds similar to those identified in Peterson, R. T., *et al., Proc. Natl. Acad. Sci. U.S.A*, *97*: 12965-12969, (2000) and Peterson, R. T., *et al. Curr. Biol.*, *11*: 1481-1491, (2001) or a panel of FDA approved chemicals may be assayed. Small molecule compounds are identified by screening large chemical libraries for the effects of compound addition to the water of developing fish. Additionally, proteins such as oligo- and polypeptides, may also act as test drugs or agents.

Further examples of such test drugs or agents include oligonucleotides or polynucleotides, such as, for example, antisense deoxyribonucleic acid (DNA), antisense ribonucleic acid (RNA), and small interfering RNAs. The antisense nucleotide sequences typically include a nucleotide sequence that is complementary to, or is otherwise able to hybridize with, a portion of the target nucleotide sequence, such as the target nucleotide sequences described herein and including the *cMYC* target nucleotide sequences (GenBank Accession No. XM_122917.1) and others described herein. The antisense nucleotide sequence may have a length of at least about 10 nucleotides, but may range in length from about 10 to about 1000 nucleotides, or may be the entire length of the gene target. The skilled artisan can select an appropriate target and an appropriate

length of antisense nucleic acid in order to have the desired therapeutic effect by standard procedures known to the art, and as described, for example, in *Methods in Enzymology, Antisense Technology*, Parts A and B (Volumes 313 and 314) (M. Phillips, ed., Academic Press, 1999).

RNA interference relates to sequence-specific, post-transcriptional gene silencing brought about by double-stranded RNA that is homologous to the silenced gene target (Lee *et al.*, *Nature Biotech.* 19:500-505 (2002)). Such a method may be used to prevent production of an oncogenic protein. Methods for inhibiting production of a protein utilizing small interfering RNAs are well known to the art, and disclosed in, for example, PCT International Application Numbers WO 01/75164; WO 00/63364; WO 01/92513; WO 00/44895; and WO 99/32619.

The test drugs or agents are typically administered in an amount and for a time necessary to suppress, or otherwise alter, or enhance oncogene-mediated neoplastic or hyperplastic transformation. Such amounts and times may be determined by the skilled artisan by known standard procedures.

Transgenic fish are typically contacted with the test drug or agent at a desired time after hatching. In other forms of the invention, the fish embryo contained with the fish egg may be contacted with the test drug or agent.

In one embodiment of the invention, determining if the test drug or agent suppresses, or otherwise alters, or enhances oncogene-mediated neoplastic or hyperplastic transformation may be performed by measuring the amount and/or size of tumors formed in the fish and/or measuring the rate of onset of tumor formation. Other indicators of oncogene-mediated, or otherwise induced, neoplastic or hyperplastic transformation, or modulation of the sensitivity of transgenic cells to treatments with radiation or chemotherapy, may also be measured. For example, when reporter gene-oncogene fusion constructs are used, reporter gene expression may be determined using methods well known by those of skill in the art and as described herein. For instance, utilizing a tissue-specific promoter operably linked to a GFP-oncogene fusion construct will permit

GFP fluorescence emitted from the protein specifically expressed in a particular tissue to be determined. Additional visual or other screens for metastatic tumors may also be used.

In some embodiments of the invention, the oncogene is a member of the *MYC* or *BCL2* gene families, and the promoter is a *RAG2* promoter. In other embodiments, the oncogene is substantially similar to, or a mammalian homologue of, *MYC* or *BCL2*.

In yet another form of the invention, methods of identifying mutations that modulate (*i.e.*, enhance, suppress, or otherwise alter) oncogene-mediated, or otherwise induced, neoplastic or hyperplastic transformation, such as the rate of onset of neoplastic or hyperplastic growth, including malignant tumors, are provided. In one form, a method involves use of genetic modifier screens. Such screens take advantage of the forward genetic capabilities of the transgenic fish described herein. Mutations that enhance the rate of onset of malignant tumors may be found, for example, in tumor suppressor genes, oncogenes or other genes involved in the neoplastic or hyperplastic process. Other mutations that alter genomic stability may also enhance the rate of onset of malignant tumors. Mutations that suppress the rate of onset of neoplastic or hyperplastic growth include, for example, proteins required for the malignant phenotype, including proteins that have not yet been identified.

In one embodiment, a method of identifying a mutation that modulates oncogene-mediated neoplastic or hyperplastic transformation includes identifying a mutation in a gene involved in the neoplastic or hyperplastic transformation process, such as a tumor suppressor gene or oncogene. Such genes may be known or unknown, and thus such methods can lead to the discovery of new tumor suppressor genes, oncogenes or other genes involved in the neoplastic or hyperplastic transformation process. In one embodiment, a mutagenized fish is crossed, or otherwise mated, with a non-mutagenized transgenic fish prone to developing cancer as described herein, to produce F_1 offspring. The method

includes obtaining eggs from F_1 females, fertilizing the eggs with inactivated sperm to produce F_2 progeny and examining the rate of onset and extent of hyperplasia, such as thymic hyperplasia, to identify F_1 fish that exhibit inactivation of a tumor suppressor gene. The method then includes identifying the mutation by methods known to the art.

In specific embodiments, transgenic fish, preferably male, homozygous for the transgene, are mutagenized and mated with a non-mutagenized, female fish homozygous for the transgene. Eggs from F_1 females are fertilized with inactivated sperm and subjected to early pressure, such as with a spindle, to produce gynogenetic diploid F_2 progeny. The rate of onset and extent of organ or tissue-specific neoplasia or hyperplasia is examined to identify F_1 fish that are heterozygous for inactivation of a tumor suppressor gene that accelerates the onset. The F_1 female can then be out-crossed to identify the mutation. The mutant allele may then be mapped and the putative tumor suppressor gene identified and cloned by standard methods known to the art.

In another embodiment, a dominant modifier screen is completed to identify genes involved in increasing, suppressing, or otherwise altering the rate of cancer induced by oncogenes in the transgenic fish described herein. The transgenic fish, as described herein, may comprise any oncogene operably linked to an organ- or tissue-specific promoter, and may or may not further comprise a reporter gene. For example, cMYC function in *RAG2-GFP* transgenic fish may be analyzed for either accelerated or delayed onset of cancer utilizing this type of screen (Figure 6). F_0 males may be mutagenized with, for example, ENU to create point mutations within the genome of the fish. These fish may then be crossed to homozygous *RAG2-GFP* female fish to create F_1 fish that are heterozygous for both the point mutations and the *RAG2-GFP* transgene. These fish may then be crossed to homozygous *RAG2-cMYC* transgenic fish and the resulting F_2 progeny can then be analyzed using methods well known by those of skill in the art. For example, GFP-positive fish may be scored for the onset of disease based on visualization of tumor formation by fluorescence microscopy

manifested by enlargement of the thymus or metastatic tumor formation in the gills, head region, and/or body musculature. While GFP expression in the T-cells is not required, it would aid in identifying early onset of disease, thereby decreasing the time needed to screen F_2 fish.

In another aspect, a method of identifying mutations that modulate sensitivity to radiation- or chemotherapy-induced programmed cell death is provided. In specific embodiments, a method comprises mutagenizing a transgenic zebrafish whose genome has stably-integrated therein a mouse *cMYC* oncogene operably linked to a zebrafish *RAG2* promoter. The transgenic zebrafish is mated with a non-mutagenized zebrafish to produce F_1 offspring. Eggs from the F_1 offspring are fertilized with inactivated sperm to produce F_2 offspring, and the presence and extent of sensitivity to radiation- or chemotherapy-induced programmed cell death in the F_2 offspring is detemined. The mutation that modulates sensitivity to radiation- or chemotherapy-induced programmed cell death is then identified.

In another specific embodiment, a method comprises mutagenizing male fish comprising the *Wik* allele to induce a point mutation in the sperm. An AB strain *RAG2-EGFP-zBCL2* transgenic female fish is also produced, and the transgenic female fish are mated with the *Wik* male fish, thereby producing F_1 progeny fish heterozygous for the *RAG2-EGFP-zBCL2* transgene and having the mutation linked to the *Wik* allele. Eggs from female F_1 progeny fish are fertilized with inactivated wild-type sperm, and early pressure is applied to the fertilized eggs, thereby producing F_2 offspring, which express the *RAG2-EGFP-zBCL2* transgene and are homozygous for the mutation. The presence and extent of sensitivity to radiation- or chemotherapy-induced programmed cell death is determined in the F_2 offspring. F_2 heterozygous mutant fish are then incrossed, thereby producing F_3 homozygous mutant fish, which comprise the *RAG2-EGFP-zBCL2* transgene and are homozygous for the mutation. The mutation in the F_3 homozygous mutant fish is then identified that modulates sensitivity to radiation- or chemotherapy-induced programmed cell death.

In yet another specific embodiment, a method comprises mutagenizing male fish comprising the *Wik* allele to induce a point mutation in the sperm. An AB strain *RAG2-EGFP-zBCL2* transgenic female fish is also produced, and the transgenic female fish are mated with the *Wik* male fish, thereby producing F_1 progeny fish heterozygous for the *RAG2-EGFP-zBCL2* transgene and having the mutation linked to the *Wik* allele. The F_1 progeny fish are then mated with the AB strain *RAG2-EGFP-zBCL2* transgenic fish, and the presence and extent of sensitivity to radiation- or chemotherapy-induced programmed cell death in the F_2 offspring is determined. The mutation in the F_2 offspring that modulates sensitivity to radiation- or chemotherapy-induced programmed cell death is then identified.

Mutations in the transgenic fish may be induced by a variety of methods, including use of various chemicals, radiation, and/or viral transduction. Such chemicals include, for example, ethylnitrosourea and DMBA. Golling *et al.* have completed viral mutagenesis screens through use of microinjection and retroviral insertion (*Nat Genet.* 31:135-40 (2002)), and this method may be used in conjunction with the various screens in the transgenic fish described herein. The sperm may be obtained by squeezing males, stimulating release of sperm, or by micro-dissection of testes. The sperm may be inactivated by UV radiation exposure.

Co-expression of two or more oncogenes may lead to accelerated onset of the disease. In the mouse model, for example, misexpression of only *TAL1/SCL* results in late-onset T-cell acute lymphoblastic leukemia (Kelliher *et al.*, *EMBO J.* 15:5160-5166 (1996); Condorelli *et al.*, *Cancer Res.* 56:5113-5119 (1996)), but when combined with the misexpression of *LMO1* or *LMO2* will lead to T-cell malignancy with a shorter latency (Larson *et al.*, *EMBO J.* 15:1021-1027; Aplan *et al.*, *EMBO J.* 16:2406-2419 (1997); (Chervinsky *et al.*, *Mol. Cell. Biol.* 19:5025-5035 (1999)). Additionally, mice transgenic for both the *Eμ-MYC* and *Eμ-BCL2* transgenes develop tumors arrested at earlier stages of B-cell development, and develop disease with shortened latency, than those in either

Eμ-MYC or *Eμ-BCL2* transgenic lines alone (Strasser *et al.*, Nature, *348*:331-333 (1990)). Cooperating mutations in the development of malignancy are common in most tumors and are typically required for cancer development.

Thus, in one embodiment, effects of oncogene co-expression are tested directly by crossing transgenic fish (*e.g.,* zebrafish) lines, as described herein, that express different oncogenes in a specific organ or tissue. Each different oncogene may be operably linked to a reporter gene, as described herein. For example, homozygous *RAG2-MYC* or *RAG2-cMYC* fish may be crossed with homozygous *RAG2-EGFP-BCL2* lines. The progeny may be analyzed for early onset of disease based on, *e.g.,* aberrant growth of GFP positive T-cells in the thymus. Other examples may include mating *RAG2-MYC* fish with transgenic fish comprising an oncogene of the *RAS, HOX11, Hox11L2, LMO1*, *LMO2* or *SCL* gene families. The outcome of such co-expression will be evident in the resultant offspring, which may be analyzed, depending on the target tissue or organ, using methods described herein and well known by those skilled in the art. For example, if transgenic fish expressing T-cell specific oncogenes are crossed, the offspring may be analyzed, for, *e.g.,* expanding populations of GFP-positive cells, T-cell histopathology, misexpression of T-cell oncogenes (*e.g.,* by *in situ* hybridization), and the presence of thymus-specific molecular markers. The rapidity of onset may be determined in cohorts of at least about 50 singly or doubly transgenic animals, as evidence for genetic cooperatively in T-cell acute lymphoblastic leukemia pathogenesis. The animals may be examined daily for the time of onset of thymic enlargement and morbidity/mortality. The survival data may then be analyzed using well-known statistical methods to assess whether the onset of disease and mortality is significantly shortened in doubly transgenic fish lines.

Reference will now be made to specific examples illustrating the transgenic fish and methods described above. It is to be understood that the examples are provided to illustrate preferred embodiments and that no limitation to the scope of the invention is intended thereby.

EXAMPLE 1: *RAG2-MYC* Transg nic Mod ls of T-c ll Leukemia

Translocation of *cMYC* gene into the T-cell receptor locus or immunoglobulin enhancer region causes dysregulation of the *cMYC* gene and results in malignant transformation of T- and B- cells in T-cell acute lymphoblastic leukemia and B-cell non-Hodgkin's lymphoma. Given that zebrafish are amenable to genetic screens, develop rapidly *ex-utero*, are transparent for much of their life cycle, and produce large clutches of offspring each week, zebrafish serve as an ideal model to study conserved pathways that lead to *MYC*-induced cancer progression in vertebrates. This example describes production of a zebrafish model of T-cell acute lymphoblastic leukemia, wherein mouse-*cMYC* was targeted to the T-cell progenitors in mosaic (or chimeric) F_0 fish, and in a stable line of zebrafish in which the progeny express and transmit to their offspring a EGFP-mMYC transgene.

Mouse cMYC and EGFP-mMYC fusion proteins were targeted to the T-cell progenitors in the developing zebrafish (Yin *et al.*, *Oncogene, 20*: 4650-4664 (2001)) through use of the zebrafish *RAG2* promoter. The *MYC* family of proteins is highly evolutionarily and functionally conserved across species (Schreiber-Agus *et al.*, *Mol. Cell Biol.*, *13*: 2765-2775 (1993); Schreiber-Agus *et al.*, Proc. Natl. Acad. Sci. U.S.A, *94*: 1235-1240 (1997); Yuan *et al.*, *Oncogene*, *17*: 1109-1118 (1998)). For example, zebrafish cMYC-I (Schreiber-Agus *et al.*, *Mol. Cell Biol.*, *13*: 2765-2775 (1993) and *Drosophila* MYC (Schreiber-Agus *et al.*, Proc. Natl. Acad. Sci. U.S.A, *94*: 1235-1240 (1997)) are capable of cooperating with activated H-RAS to effect the malignant transformation of mammalian cells, indicating that these proteins are functionally similar across phyla and that cMYC proteins from different species have similar transformation properties when placed into human cell lines.

A. Materials and Methods

1. DNA Constructs

The *RAG2-GM2* plasmid was digested at 37°C with BamH1 and HindIII overnight to release the GM2 cassette. The enzyme digest mix containing the linearized plasmid vector was heat-inactivated at 80°C, cooled to 4°C, and phosphorylated overnight with alkaline phosphatase at 37°C. The following day, the reaction was heat-inactivated and stored at 4°C until use.

mcMYC and *EGFP-mcMYC* were ligated into the linearized *RAG2*-vector. Specifically, PCR was used to amplify the mouse *cMYC* open reading frame. The forward PCR primers contained a 6 bp leader sequence (AATTCC) followed by a BamH1 and Sma1 cloning site, 6 bp 5' the ATG start site (presumably specifying the Kozak sequence) and 17 bp of coding sequence, including the ATG start site. The reverse primers contained 6 bp of leader sequence, a HindIII cloning site, and 24 bp of coding sequence, including the termination codon sequence. PCR-amplified fragments were digested overnight with BamH1 and HindIII at 37°C and resolved on an ethidium bromide-containing agarose gel (1% in 1xTBE). Gel bands were excised and DNA extracted by QIAGEN QIAquick® gel extraction kit (QIAGEN, Valencia, CA). The DNA was ligated into the linearized *RAG2* vector overnight at 4°C and used for transformation into competent JM109 bacteria cells (Promega, Madison, WI). Cells were grown on ampicillin-containing agarose plates, and positive clones were identified by colony PCR using *cMYC*-specific primers. Positive clones were grown in LB + ampicillin overnight, and the plasmid prepped (Wizard® SV Minipreps, Promega, Madison, WI).

RAG2-EGFP-MYC constructs were made using linker-mediated ligation. Specifically, the *EGFP-MYC* vector was digested with Nhe1 and EcoR1 to release the portion of the vector containing the EGFP-*MYC* coding sequence. This fragment was ligated into the linearized, alkaline phosphatase-treated PCS2 vector, (digested with BamH1 and EcoR1) in the presence of a phosphorlyated double strand linker. The linker adapted the BamH1 overhangs from the PCS2 vector into Nhe1 compatible ends. These *PCS2-EGFP-MYC* vectors were digested with BamH1 and HindIIII, to release the coding sequence and ligated

into the linear *RAG2*-construct as above. Both of these methods for cloning into the *RAG2* vector are methods well known by those of skill in the art.

Additionally, a *RAG2*-human *MYC-ER* construct was created using a PCR based cloning strategy (as above). The *MYC-ER* transgene is a fusion between the human *cMYC* gene and a portion of the estrogen receptor (ER) responsible for binding estrogen. This fusion transgene has been reported to be conditionally activated in mice. In the absence of tamoxifen (an estrogen analog), MYC-ER is retained in the cytoplasm and is not functional. In the presence of tamoxifen, MYC-ER translocates to the nucleus where it is able to activate genes involved in MYC-induced transformation. Tamoxifen may be administered to mice in their drinking water and results in the activation of the *MYC-ER* allele. A similar transgenic system may also be use in fish, wherein the addition of tamoxifen to the water will activate the *MYC-ER* alleles in transgenic fish.

Finally, a loxed *dsRED2* allele was cloned into the *RAG2-EGFP-mMYC* plasmid at the BamHI site, upstream of *the EGFP-mMYC* transgene. The resulting *RAG2-Lox-dsRED2-EGFP-mMYC* plasmid contains lox sites which flank the *dsRED2* transgene (Figure 7.A). In the absence of CRE recombinase, the *dsRED2* transgene is expressed, while the *EGFP-mMYC* transgene is not active (Figure 7.B-7.C). In the presence of CRE-recombinase activity, the *dsRED2* allele will be excised placing the *RAG2*-promoter adjacent to the *EGFP-mMYC* transgene. This will result in expression of EGFP-mMYC in thymic progenitors, leading to the development of T-cell Leukemia/lymphoma in transgenic fish.

To verify that this CRE/Lox-mediated strategy worked in fish, we created an expression vector which expresses the *dsRED2* loxed allele under control of the ubiquitous expressing CMV promoter containing the *EGFP* transgene (figure 8.A). The resulting plasmid contains lox sites that flank the dsRED2 transgene (as outlined above), which lies upstream of a *EGFP* transgene. As in the *RAG2-Lox-dsRED2-EGFP-mMYC*, *dsRED2* is expressed in the absence of CRE

induction, while EGFP expression is silenced. Upon CRE-induction, the *dsRED2* allele is excised, juxtaposing the CMV promoter next to the EGFP transgene. This results in expression of *GFP* following CRE-mediated recombination. A vector containing CMV promoter driving expression of CRE was also established to deliver CRE recombinase activity to embryos during early development (PCS2+CRE).

2. Embryo injections: Transient analysis of CRE recombination in fish.

To verify that the CRE-recombinase strategy works in the zebrafish, we co-injected the *CMV-lox-dsRED2-EGFP* plasmid with either the PCS2+CRE plasmid DNA or with the *CRE* mRNA made from this plasmid. Specifically, the PCS2+CRE vector was linearized by Not1 digestion overnight, and the resulting cleaved DNA was phenol:chloroform/ethanol precipitated. Because the PCS2+ vector contains an *Sp6* promoter site 5' of the *CRE* transcription start site, we were able to make *CRE* RNA using an *SP6* RNA polymerase. RNA was purified using a lithium chloride extraction and resuspended to a final concentration of 200 ng/microliter.

For DNA:DNA injections, a solution containing 50 ng/microliter of *CMV-lox-dsRED2-EGFP* plasmid and 50 ng/microliter of PCS2+CRE plasmid was injected into embryos at the one-cell stage of development. For DNA:RNA injections, a solution containing 25 ng/microliter of *CMV-lox-dsRED2-EGFP* plasmid was injected with 50 ng of *CRE* RNA. Embryos were analyzed at 24 and 48 hours for expression of GFP and dsRED2 as determined by fluorescent microscopy.

3. Embryo injections: Creation of mosaic founder fish and stable transgenic lines.

RAG2-cMYC, RAG2-EGFP-MYC, RAG2-hMYC-ER, and RAG2-lox-dsRED2-EGFP-mMYC vectors were linearized by digestion with Xho1 or Not1 at

37°C overnight, and DNA was purified by phenol:chloroform extraction followed by ethanol precipitation. DNA concentration was assessed by both UV spectrophotometry and gel quantification. Linearized DNA was diluted to 100-200 ng/microliter in 0.5xTE/100 mM KCl and injected into wild-type AB embryos at the one-cell stage of development using a glass micropipette. The volume of DNA injected was tittered to a point at which 20-50% of the embryos exhibited morphological features associated with over-injection of DNA. This is commonly seen in microinjection into zebrafish and ensures that surviving embryos have high levels of DNA integration into cells. The 20-50% of embryos exhibiting "monster" morphology were discarded. Embryos were grown in E3 egg water at 28.5°C until 15 days of development, at which time they were placed into our re-circulating system. These F_0 mosaic fish were analyzed daily for onset of tumors based on external morphologic features, which are characteristic of lymphoma, including thymic enlargement (protrusion of the thymus from the opercul), and tumor formation in the eyes or head.

Microinjection of *RAG2-cMYC* was also completed in AB embryos heterozygous for the *RAG2-GFP* to visualize tumor formation by fluorescence microscopy. *RAG2-GFP* stable transgenic lines had been established previously. Tumor onset was assessed by aberrant expression of GFP within mosaic *RAG2-mcMYC* fish by fluorescence microscopy once each week.

4. Histological methods.

Tumorigenic fish and AB control fish were analyzed histologically. Fish were fixed in 4% paraformaldahyde at 4°C overnight and processed for embedding in paraffin. Methods for dehydration of tissue samples is well known to those skilled in the art. Fish were cut transversely into 5-10 mm sections and embedded in paraffin. Blocks were sectioned at 5-10 microns through the length of the fish and every tenth slide was stained with hematoxylin/eosin. Slides were analyzed by light microscopy for tumor formation as compared to wild-type AB slides.

5. *In situ* hybridization.

Methods for RNA *in situ* hybridization on paraffin embedded slides is well known to those skilled in the art (*e.g.,* Wilcox *et al., In situ* cDNA:mRNA hybridization: Development of a technique to measure mRNA levels in individual cells. IN: Methods in Enzymology, Vol. 124, Neuroendocrine Peptides (P.M. Conn. Ed.) Academic Press, pp. 510-533 (1986); Rosenthal *et al., EMBO J.* 6:3641-3646 (1987); Wilcox *et al., J Neurosci.* 8:1901-4.1988; Wilcox *et al., Mol Cell Biol.* 8:3415-22. (1988), Melton *et al., Nucleic Acids Res.* 12:7035-56 (1984); Maniatis *et al., Molecular Cloning: A Laboratory Manual,* 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1982); and Yang *et al., J Histochem Cytochem.* 47:431-46 (1999)), and was completed with only minor variations. Briefly, cDNA probes were made by PCR of plasmid DNA containing coding sequences for genes of interest, including *cMYC, SCL, LMO1, LMO2, RAG1, RAG2, TCRα, LCK, IgLC,* and *IgM.* PCR primers included *T7* and *Sp6* promoter sequences and were incorporated into the amplified fragments. PCR products were 400-600 bp in length and were purified using the QIAquick® PCR purification kit (QIAGEN, Valencia, CA). RNA probes were made using T7 and Sp6 RNA polymerase. Hybridization was carried out, and RNA staining was visualized using peroxidase staining techniques.

6. Southern analysis.

Southern analysis was completed as described by Haire *et al. Immunogenetics 51*: 915-923 (2000) with some variations. Specifically, genomic DNA was isolated from the posterior portion of the fish. Fish were anesthetized and cut in half transversely at the anus. Genomic DNA from the tail portion of the fish was extracted (PUREGENE™ DNA Isolation Kit, Gentra Systems, Minneapolis, MN), quantified by spectrophotometric analysis, and 8 mg of DNA was digested overnight at 37°C with the BglII restriction enzyme. DNA fragments were electrophoresed on a 0.8% agarose gel (1x TBE) containing ethidium bromide for 4 hours. DNA was transferred to nylon membranes and the blots

were probed with radiolabelled *TCRα* or *IgM* probes. These probes were specific to the constant regions of these genes and were made by random-primed labeling (Rediprime™ II, Amersham Biosciences, Piscataway, NJ). Washing of membranes was completed under high stringency conditions and membranes were exposed to x-Ray film for 1-3 days, after which time they were developed.

7. FACS analysis.

Kidney cells and spleen cells were isolated from wild-type AB fish or from mosaic *RAG2-MYC/EGFP-MYC* fish following dissection. Harvested cells were resuspended in 0.9xPBS and 5% FBS, separated by a 40 micron filter, washed, and stained with propidium iodine. FACS was completed based on forward and side-scatter, and in some instances based on GFP fluorescence.

8. Blast morphology.

Cytospins were completed on whole kidney marrow and whole spleen as well as on FACS sorted cell populations. Cells were stained with May-Grunwald, cover slipped, and viewed under high power.

9. Touch preps.

Fish were anesthetized and cut in half transversely at the anus. The cut portions of the fish were placed onto glass slides repeatedly (*i.e.* a 'touch prep'). Slides were dried from several minutes and stained with May-Grunwald as described above.

10. PCR verification of transgene incorporation into genomic DNA.

Fish were anesthetized and cut in half transversely at the anus. The tail portion was extracted for genomic DNA by either PUREGENE™ protocols (Gentra Systems, Minneapolis, MN) or Proteinase-K digestion as outlined in *The Zebrafish Book: A Guide For the Laboratory Use of Xebrafish (Danio rerio),* 4th Ed. (M. Westerfield *ed.*, University of Oregon Press, Eugene, OR (2000)). PCR

was completed using primers spanning both the *RAG2*-promoter and the *MYC* coding sequence. The forward primer was within the *RAG2*-promoter sequence while the reverse primer was within the *MYC* coding sequence. Control primers amplified across the *RAG2* promoter and *RAG2* open reading frame.

11. DNA flow cytometry.

Body musculature obtained from the posterior portion of tumorigenic fish was diced over ice-cold 0.9xPBS and 5% FBS, separated by a 40 micron filter, washed, and stained with propidium iodine. Kidney cells from wild-type fish were harvested by dissection and resuspended in 0.9xPBS and 5% FBS, separated by a 40 micron filter, washed, and stained with propidium iodine. Twenty five hundred cells were analyzed by DNA flow cytometry. Combinations of tumor and wild-type cells verified that peaks identified in tumor samples were 2N or heterodiploid.

12. Semi-Quantitative RT-PCR.

RNA was isolated from *RAG2-EGFP-mMYC* stable transgenic offspring using a Trizol extraction protocol. RNA was quantified and made into cDNA using a reverse transcriptase reaction and diluted 1:1, 1:10, 1:100, and 1:1000 with water. Dilutions of cDNA were subject to PCR amplification in the presence of primers specific to zebrafish *LMO1, LMO2, HOX11, TLX-3a, TLX-3b, SCL, LCK*, and mouse *cMYC*.

B. The RAG2-cMYC, RAG2-EGFP-cMYC, and RAG2-human MYC-ER constructs drive tumor formation in F_0 chimeric fish.

Of 215 F_0 *RAG2-mcMYC* F_0 founders, 11 fish exhibited external phenotypes associated with extensive tumor formation, while 7 of 122 *RAG2-EGFP-mcMYC* fish exhibited signs of disease. The mean latency of tumor onset was 45 days and 52 days for *RAG2-mcMYC* and *RAG2-EGFP-mcMYC* fish,

respectively. The mean survival, the time at which fish were determined to be terminally ill and were sacrificed, was 64 days for both constructs. Tumor onset ranged from 30 to 131 days. The longest surviving tumor-bearing fish lived 178 days, at which time it was sacrificed.

Some of the *RAG2-MYC-ER* chimeric fish developed tumors in the absence of tamoxifen-induction (4 of 178 by day 114, 2.2%), indicating that the fusion transgene is active, but somewhat "leaky." Of the *RAG2-MYC* fish, 5.3% (18 of 337) developed leukemia, with a mean latency of 51 days (range 30-131d). Only 2.2% of the *RAG2-MYC-ER^{Tm}* chimeric fish had tumors by 114 days. Thus, the use of the ER-conditional system is promising. Similar results were seen in *CD2-ER-MYC^{Tm}* mouse models, where up to 23% of offspring develop tumors in the absence of tamoxifen, while 62% develop tumors by 300 days of development in the presence of tamoxifen (Blyth *et al.*, 2000).

Leukemic fish that were mosaic for *RAG2-mMYC, RAG2-EGFP-mMYC, and RAG2-MYC-ER* expression had severely distended abdominal cavities, splayed eyes, and variable tumor formations found between and around the eyes (Figures 1A-1F). Some fish had growths protruding from the operculum in a position likely corresponding to the thymus (Figures 1E-1F) and tumors were commonly found at the base of the pectoral fin (Figures 1C-1D). Many of the fish had tumors just beneath the skin, resulting in fish becoming less pigmented over time. In contrast, no *RAG2-Lox-dsRED2-EGFP-mMYC* chimeric fish developed leukemia/lymphoma.

C. Analysis of tumor formation in transgenic fish.

Three *RAG2-mcMYC* F_0 fish and four *RAG2-EGFP-mcMYC* fish were sectioned and analyzed for tumor formation based on hematoxylin/eosin staining. All fish analyzed had massive tumor burden with extensive tumor formation occurring throughout the body (Figures 1I-1P). Massive infiltration of lymphocytes into the liver, spleen, and kidney (the equivalent of the mammalian

blood marrow) was observed (Figure 1L). Tumor cells were found in between muscle fibers in the dorsal musculature (Figure 1N), gut, gills, fins, areas surrounding the eye, and just bellow the skin. Prominent tumors were noted in the nasal region (Figure 1P) that did not appear to arise from RAG2-expressing epithelial cells of the olfactory bulb. Instead, tumors in the nasal region were *LCK*-positive by *in situ* analysis and failed to stain with keratin antibody (data not shown), indicating that these nasal tumors were the same T-cell tumors noted throughout the fish. The *RAG2-EGFP-mcMYC* F_0 fish (n=4) had tumors similar to those described for *RAG2-mcMYC*, although GFP could not be detected by fluorescence microscopy or FACS analysis. Given that the half-life of cMYC is about 30 minutes in normal cells and that the maturation time for the GFP protein is approximately 4 hours, it is not surprising that GFP was not detected, as most of the fusion protein would be targeted for destruction before GFP maturation and fluorescence could occur.

Of the seven tumor-bearing fish analyzed histologically, all had T-cell acute lymphoblastic leukemia. Paraffin sections were analyzed for RNA expression of the T-cell genes *TCRα* and *LCK* and the B-cell genes *IgM* and *IgLC*. The lymphoid markers *RAG1* and *RAG2*, and *mcMYC* expression was analyzed by *in situ* hybridization. All tumors were positive for *mcMYC* (Figures 2A-2B), *RAG1*, *RAG2* (Figures 2C-2D), and *LCK* (Figures 2E-2F). RNA expression of the other genes could not be detected. *RAG2-EGFP-mcMYC* fish were analyzed for expression of the GFP-fusion protein by immunocytochemistry using the anti-GFP antibody (Clontech, JL-8) and showed strong nuclear protein expression in tumor cells (Figures 2G-2J). These results indicate that the *RAG2-* promoter drives both *mcMYC* RNA and protein expression, and is responsible for oncogenic transformation of T-cells in the mosaic F_0 fish.

Two *RAG2-mMYC* and two *RAG2-EGFP-mMYC* F_0 mosaic fish were analyzed by FACS analysis and total cell counts were made for the kidney and spleen. In wild-type AB fish, the kidney typically has 8.4 +/- 3.7 x 10^5 (N=7) blood cells, while the spleen has 8.2 +/- 6.3 x 10^4 (N=6) (Traver *et al.*, in

preparation). In the tumor bearing fish, 4.95 +/- 3.3 x 10^6 (N=5) cells were found in the kidney and 4.64 +/- 1.5 x 10^5 (N=4) cells were found in spleen. Although the leukemic kidneys and spleens contained more cells in total than wild-type controls, total red blood cell and myeloid population numbers were not drastically altered, indicating that lymphocytes accounted for the increased number of cells found in the spleen and kidney (Figure 3). The percentage of blasts in the kidney was 87.1 +/- 3.9% (N = 4, range 83.1 to 92.2%), while in the percentage of blasts in the spleen was 75.7 +/- 10.5% (N=4, range 60.8 to 85.3%). Tumor cells had a granularity similar to that of normal lymphocytes based on side scatter (SSC on Y-axis of Figures 3A-E), but were slightly larger based on forward scatter (FSC on X-axis). Because the kidney marrow is analogous to the bone marrow in mammals, and because leukemia is generally defined as >30% blasts in the bone marrow, the mosaic fish had disseminated T-cell acute lymphoblastic leukemia with blasts accounting for 87.1+/-3.9% of the cells in the kidney.

D. Verification of clonality and lineage of other tumors.

To determine clonality as well as lineage of the tumor cells, Southern blot analysis was performed on restriction enzyme-digested tumor DNAs using radiolabeled probes that corresponded to the zebrafish *TCRα* (Haire *et al., Immunogenetics, 51*: 915-923 (2000)) and *IgM* constant regions. Of the three tumors analyzed, one had monoclonal *TCRα* gene rearrangements and one had oligoclonal *TCRα* gene rearrangements (Figure 2I). The third tumor showed germ line configuration of *TCRα* but had very strong *LCK* RNA expression, which confirmed T-cell origin. Tumor cells from the third tumor were typically arrested in thymic differentiation prior to *TCRα* gene rearrangement. None of the tumors had rearrangement of the *IgM* gene, which, when combined with the presence of *TCRα* gene rearrangements and *LCK* gene expression, indicated that all the tumors identified were of thymocyte origin.

Mice that expressed *cMYC* under the control of *CD2* or *Thy1* promoters also developed T-cell lymphoma, which arose from monoclonal or oligoclonal

populations of T-cells. Thus, the fish and mouse tumors were similar with respect to stage of differentiation arrest and clonality.

E. Transplantation of tumor to recipient fish.

To analyze whether tumors could be transplanted, tumor cells were harvested from the kidneys of F_0 mosaic fish and injected intraperitoneally into 2-day old AB wild-type embryos. Of 25 fish injected, one fish developed leukemia by day 44 (Figure 4A). This fish had a similar phenotype as that observed in F_0 mosaic fish, having a distended abdomen, splayed eyes, and tumors extending from the operculum to the pectoral fin joint. Touch preps confirmed that this transplanted fish had tumors similar to F_0 mosaic fish (Figures 4B-D), as did histological analysis (data not shown). PCR was completed on tumor DNA, which was extracted from the invading cells in the tail region, and confirmed that the fish carried the *cMYC* transgenic construct (Figure 4E).

Tumors were also transplantable into irradiated adult fish. Fish were anesthetized with tricaine and sublethally irradiated with 2500 Rads from a ^{35}Cs source two days prior to injection with tumor cells. Five fish were injected intraperitoneally with either 3.6×10^5 total kidney cells or 1×10^5 sorted blasts. Of the five fish injected with whole kidney marrow, one died as a result of the injection procedure, and two died before the analysis could be completed. The remaining two fish were sacrificed 25 days after injection and analyzed histologically for tumor formation. Both fish had massive infiltration of tumor cells into the kidney (Figure 4G) and body musculature (Figure 4I) when compared to uninjected, irradiated control fish (Figure 4F and 4H). Of the five fish injected with sorted blasts, three survived until 25 days post-injection, at which time they were sacrificed. All three fish had tumor formation similar to both the F_0 mosaic fish and the fish injected with whole kidney marrow. However, two of the fish had less severe tumor invasion when compared to other transplanted fish, possibly as a result of less cells being injected into fish receiving sorted blast cells. RNA *in situ* hybridization confirmed that transplanted

tumors expressed *mMYC*, *RAG1*, and *RAG2* and were morphologically indistinguishable from tumors arising in F_0 mosaic *zRAG2-mMYC* fish (data not shown), All three control irradiated fish analyzed had normal histological morphology when compared to non-irradiated control fish.

F. Determination of ploidy of *RAG2-mcMYC* tumors.

MYC expression in tumors often results in genomic instability and accumulation of extrachromosomal fragments. To determine if *RAG2-mcMYC* tumors were heterodiploid, FACS analysis was completed on one tumor-bearing fish to determine the DNA content in the tumor cells (Figure 4B). The analyzed tumor had a DNA index of 1.15. Upon mixing tumor and wild-type sample, the tumor peak decreased while the 2N peak increased (data not shown), verifying that the 2N and tumor peaks were distinct in tumor samples.

G. Developing stable lines of transgenic zebrafish that express the RAG2-EGFP-mMYC transgene.

Although 5% of the F_0 mosaic fish develop lymphoma, other injected F_0 fish may have transgene integration into germ cells without integration into the lymphoid cells. These fish should be healthy and should give rise to transgenic F_1 progeny. Offspring from ~350 F_0 founders were screened by genomic PCR, and 7 individuals were identified that were able to pass on the transgene tot he F_1 progeny. Of those, only one expressed the *EGFP-MYC* transgene. RAG2-EGFP-MYC F_1 fish progeny of this founder fish developed GFP-positive thymic tumors by 22 days of development (Langenau *et al.*, 2003).

These tumors expressed the zebrafish *RAG1*, *RAG2*, and *Lck* genes, which indicated that the tumors that developed in the *RAG2-EGFP-mMYC* stable line were similar to those that developed in mosaic fish. Additionally, tumors had clonal rearrangement of the TCRα genomic locus, and some tumors were clonally aneuploid as determined by DNA content. Five of 34 (15%) tumors analyzed had DNA indices ranging from 1.02-1.10. Finally, *LMO2* and *SCL* were expressed in our *EGFP-mMYC*-induced leukemias, as confirmed by both RT-

PCR (Figure 10) and *in situ* analysis (Figure 11). The most common subclass of childhood T-ALL expresses both *SCL* and *LMO1/2*, and this subclass of patients has the worst prognostic outcome of all patient groups. Thus, this zebrafish model may be used for the most common and most deadly form of human childhood T-ALL.

Given the early onset of tumor formation in F_1 *RAG2*-transgenic fish that harbor *EGFP-MYC* transgene, and that the mean survival of transgenic fish is 81 days (range 50 to 111 days, n = 53), it has not been possible to mate F_1 fish. However, because the testes are developed by 8-12 weeks of age, the testes from leukemic male fish have been extracted for use in *in vitro* fertilization to maintain the lines. The line has been carried and F_3 fish were produced and are currently growing in our system. Because the IVF procedure is cumbersome, and may not be amenable for use in large scale genetic screens, conditional transgenic zebrafish lines using a tamoxifen-sensitive *MYC-ER^{Tm}* transgene (Blyth *et al.*, 2000) will be generated, and a CRE-mediated strategy will be pursued to induce expression of *the EGFP-mMYC* transgene, as outlined above (Example 1.A.1).

H. Developing conditional stable lines of transgenic zebrafish that express an inducible MYC transgene.

At least two different strategies may be used to generate conditional transgenic zebrafish lines that express inducible *MYC* alleles under control of the *RAG2* promoter.

The first strategy utilizes the tamoxifen-inducible human *MYC-ER* fusion transgene. Approximately 400 fish were injected with the *RAG2- MYC-ER^{Tm}* transgene and are currently growing up in our system. Some of these fish developed tumors (4 of 178 by day 114, 2.2%), which indicated that the transgene is active, but somewhat "leaky." Of the *RAG2-MYC* fish, 5.3% (18 of 337) developed leukemia, with a mean latency of 51 days (range 30-131d). Only 2.2% of the *RAG2-MYC-ER^{Tm}* chimeric fish developed tumors by 114 days. Thus, the use of the ER-conditional system is promising. Similar results were

seen in *CD2-MYC-ER*^{Tm} mouse models, where up to 23% of offspring developed tumors in the absence of tamoxifen, while 62% develop tumors by 300 days of development in the presence of tamoxifen (Blyth *et al.*, 2000).

F_0 fish that yield PCR-positive offspring will be mated weekly, and their clutches will be raised. Some clutches will be used to verify that the transgene is expressed in the offspring by either whole mount *in situ* hybridization or by immunocytochemistry using antibodies specific for human cMYC or the estrogen receptor. The remaining clutches will be raised to adulthood, and DNA will be isolated from fin clips for PCR screening to identify F_1 founders.

To test the response of MYC-ER^{Tm} to tamoxifen in the fish, the *RAG2-MYC-ER*^{Tm} transgene will be injected into embryos from the *RAG2-GFP* line, raised for 6 days in the absence of tamoxifen, and scored for GFP-positivity. Embryos with GFP-positive T-cells by 6 days of development will be raised in the presence of tamoxifen (0, 0.1, 1.0, and 10 μM) from day 6 to day 35, and scored for tumor development at 35 days. Water changes will be completed every 3 days over this period with tamoxifen being replaced each time. These experiments will define what dose of tamoxifen is necessary to induce the MYC-ER^{Tm}. Because 5% of injected fish integrate the transgene into their T-cells (Langenau *et al.*, 2003), at least 100 primary injected fish will be assessed per group. To verify that the response is due to tamoxifen and not the "leakiness" of the *MYC-ER*^{Tm} transgene, leukemic fish will be removed from tamoxifen and scored for tumor regression.

The second strategy to produce conditional transgenic zebrafish lines uses a CRE/Lox-mediated excision event to drive expression of the *EGFP-mMYC* transgene. We co-injected the *CMV-Lox-dsRED2-EGFP* plasmid in combination with CRE plasmid or *CRE* RNA. In embryos injected with *CMV-Lox-dsRED2-EGFP* plasmid alone, fish were red fluorescently labeled, but not green. In contrast, embryos co-injected with plasmid, the fish were both red and green under fluorescence, which indicated that CRE-recombination occurred in the zebrafish embryos and that the plasmids were co-expressed in some but not all cells (Figure 8.B-8.E). To test efficiency of CRE excision of the *dsRED2* loxed

allele, we co-injected *CMV-Lox-dsRED2-EGFP* with or without *CRE* RNA. In the absence of *CRE* RNA, fish were red, but not green (Figure 9.A-9.C). When 50 ng/μl of *CRE* was co-injected with the *CMV-Lox-dsRED2-EGFP* plasmid, there was 100% excision of the *dsRED2* allele; the fish were green, but never red (Figure 9.D-9.F). These results indicated that the CRE/lox strategy worked transiently in the fish, and highlighted the fact that *CRE* RNA was not toxic to fish and may be injected *in vivo*.

A *RAG2-loxed-dsRed2-EGFP-MYC* was made and injected into over 300 surviving fish. The transgene was detected by dsRed2 fluorescence at 6 days post-fertilization (Figure 7.B and 7.C). Mosaic fish were identified, which have strong expression of the red fluorophore in T-cells at three months of age, However, none of these fish developed tumors, which indicated that the ds-Red protein was being expressed, while the EGFP-MYC protein was not. The F_0 founder fish will be screened for the ability to pass on the transgene, as detected by dsRed2 fluorescence.

I. Discussion.

Understanding the genetic events that lead to tumor formation in cMYC-expressing cells is important to developing treatments for diseases such as Burkitt's Lymphoma, follicular B-cell lymphoma, and T-cell acute lymphoblastic leukemia. By identifying the genetic events that stop transformation in fish prone to developing cMYC-induced tumors, it may be possible to identify conserved mechanisms involved in human disease. The genes identified by genetic suppressor screen are potential targets for drug development and may lead to new therapies in diverse cancers in which *cMYC* is amplified or overexpressed. Additionally, small molecule inhibitors or other drugs or agents identified in chemical screens that suppress *MYC*-induced leukemia in the transgenic fish described herein fish may be candidates for clinical trials in human patients and may lead to the discovery of new therapeutic agents in the future. Finally, developing a model of cancer in the fish provides a precedent for developing

other models of malignant transformation in the fish that utilize other promoters and other oncogenes, some additional examples follow.

EXAMPLE 2: Use of *RAG2-EGFP-zebrafish BCL2* Transgenic Models to identify chemical and genetic suppressors of BCL2 function in T-cells.

A. Materials and Methods.

Diseases such as follicular B-cell lymphoma have activation of *BCL2,* an anti-apoptotic gene, which renders these tumors resistant to apoptosis and allows them to become less sensitive to chemotherapy and radiation therapy than B-cells transformed by other mechanisms. Additionally, a large proportion of tumors misexpress the *MYC* oncogene, a transformation event that necessarily requires inactivation of the apoptotic machinery. Creating drugs that inhibit BCL2 or MYC function in transformed cells would be advantageous for treating human tumors and may be used in combination with chemotherapy and radiation therapy to selectively kill cells that have overexpression of this oncogene. Similar experiments to those described herein for *BCL2* may also be performed with, *e.g., cMYC.*

1. DNA constructs.

Degenerate PCR was used to amplify a zebrafish *BCL2*-related cDNA fragment from 1-5 day embryos, and the resulting DNA fragment was cloned into a pGEMT-EASY vector (Promega). Clones were sequenced and used as probes to obtain a full-length clone by screening a whole embryo cDNA library (RZPD). Specifically, the plasmid DNA was digested to release the *BCL2*-related cDNA fragment, resolved on an ethidium bromide-containing agarose gel, and DNA was extracted from the gel band. The gel purified DNA was radiolabled using the REDI-PRIME II random labeling system (Amersham) and used to probe a full-length cDNA library. One full-length clone was obtained and sequenced.

Deduced amino acid similarity of zebrafish BCL2 (zBCL2) placed our gene with other *BCL2* family members (Figure 12). Phylogenetic analysis suggested

that our zebrafish *BCL2*-like gene was likely the true *BCL2* homolog in fish, as it clustered with other vertebrate BCL2 proteins and failed to cluster with BCL-xL proteins.

We created the *EGFP-zBCL2* gene-fusion by fusing *EGFP* to the amino terminus of *zBCL2* (Figure 13.A). Specifically, the *zBCL2* cDNA was amplified using PCR in the presence of a forward primer that altered the methionine start site. Additionally, the forward primer contained a Sal1 site, while the reverse primer contained a HindIII site to facilitate cloning into the *EGFP-C1* vector (Clonetech). The resulting PCR fragment was PCR purified using the QIAQUICK PCR purification system (Qiagen) and digested overnight with Sal1 and HindIII. The digested PCR product was resolved on an ethidium bromide-containing agarose gel. The resulting DNA band was excised, and the DNA was extracted. This DNA was cloned into the *EGFP-C1* vector, which had been digested with Xho1 and HindIII. The resulting plasmid created an *EGFP-zBCL2* gene fusion.

Next, the *RAG2-EGFP-zBCL2* transgene was made by digesting the above vector (*EGFP-C1-zBCL2*) with BamHI and HindIII, and the resulting fragment was cloned into the *RAG2*-promoter-containing vector, essentially as outlined in Example 1.A.1. This *RAG2-EGFP-zBCL2* vector was then digested with Cla1 and BamH1, and cloned into the pCS2+ vector.

2. Embryo injections: Transient analysis of the EGFP-zBCL2 transgene in fish.

To verify that our fusion protein was functional, *EGFP-zBCL2* RNA and GFP control RNA was injected into embryos at the one-cell stage. Embryos were then analyzed for inhibition of developmentally regulated apoptosis at 16, 20, and 24 hours. Additionally, GFP-positive *EGFP-zBCL2* RNA and *GFP* control RNA-injected embryos were irradiated with 16 Gy of irradiation at 14 hpf and fixed in paraformaldahyde at 20 hpf. RNA was created and injected as outlined essentially as described for PCS2+CRE RNA in Example 1.A.2. GFP-positive fish were sacrificed and analyzed for the presence of apoptotic cells by TUNNEL assay.

3. Whole mount tunnel analysis.

Whole mount TUNEL analysis was completed using TMR-Red *In Situ* Cell Death Kit (Roche).

4. Embryo injections: Creation of mosaic founder fish and stable transgenic lines.

The *RAG2-EGFP-zBCL2* vector was linearized by digestion with Xho1 or Not1 at 37°C overnight, and DNA was purified by phenol:chloroform extraction followed by ethanol precipitation. DNA concentration was assessed by both UV spectrophotometry and gel quantification. Linearized DNA was diluted to 100-200 ng/microliter in 0.5xTE/100mM KCl and injected into wild-type AB embryos at the one-cell stage of development using a glass micropipette. The volume of DNA injected was titered to a point at which 20-50% of the embryos exhibited morphological features associated with over-injection of DNA. This is commonly seen in microinjection into zebrafish and ensures that surviving embryos have high levels of DNA integration into cells. The 20-50% of embryos exhibiting "monster" morphology were discarded. Embryos were grown in E3 egg water at 28.5°C until 15 days of development, at which time they were placed into a re-circulating system.

5. Histological Methods.

RAG2-EGFP-zBCL2 transgenic and AB control fish were analyzed histologically. Fish were fixed in 4% paraformaldehyde at 4°C overnight and processed for embedding in paraffin. Methods for dehydration of tissue samples are well known in the art. Fish were cut transversely into 5-10 mm sections and embedded into paraffin. Blocks were sectioned at 5-10 microns through the length of the fish and every tenth slide was stained with hematoxylin/eosin. Slides were analyzed by light microscopy and compared to wild-type AB fish sections.

6. Irradiation sensitivity.

RAG2-GFP and *RAG2-EGFP-zBCL2* transgenic fish were treated with a whole-body dose of 15, 20, and 25 Gy of ionizing irradiation. Fish were analyzed for irradiation responses at 6 dpf, 21 dpf, and 3 months of age as determined by loss of GFP labeled T-cells in the thymus.

B. The EGFP-zBCL2 transgene is functional.

To verify that our fusion protein was functional, *EGFP-zBCL2* RNA was injected into embryos at the one-cell stage. Embryos were analyzed for inhibition of developmentally regulated apoptosis at 16 hours post-fertilization. GFP-positive fish were sacrificed and analyzed for the presence of apoptotic cells by TUNEL assay. The *EGFP-zBCL2* RNA rescued apoptosis that normally occurred in the developing embryo (Figure 13.B-G.D). Additionally, *EGFP-zBCL2* RNA rescued irradiation-induced apoptosis in the developing embryos (Figure 14). Both of these experiments showed that the *EGFP-zBCL2* transgene was a potent inhibitor of apoptosis *in vivo,* and the transgene may be easily visualized in living fish.

C. T-cells which express the RAG2-EGFP-zBCL2 transgene are protected from irradiation-induced apoptosis.

T-cells from *RAG2-EGFP-zBCL2* transgenic fish did not undergo apoptosis following treatment with ionizing radiation (15, 20, and 25 Gy), as determined by continued GFP expression in the thymus. In contrast, *RAG2-GFP* transgenic fish lost GFP-labeled T-cells 2 to 3 days post-irradiation treatment in both 8 day-old fish (Figure 15) and 3 month-old fish (Figure 16). The response to radiation was an "all or nothing" response. Thus, scoring the phenotypic changes marked by GFP-expression in the thymus an easy assay for apoptosis in the T-cells at 8 and 23 days of age, and in the T-cells at 3 months of age.

D. Developing a high-throughput 96-well assay.

Next it was determined if 5- to 8-day old zebrafish embryos are able to survive in 96 well plates, and what concentration of water was necessary to

attain high levels of survival. Six-day old zebrafish embryos were allocated to wells in a 96 well plate. Three to four embryos were grown in 250, 150, and 100 µl of egg water with and without 1% DMSO and analyzed daily for mortality.

E. A 96-well assay for drug treatment.

5- to 8-day old zebrafish embryos were able to survive in as little as 100 µl of egg water for up to 2 days post-treatment. However, significant mortality was seen in wells containing 100 µl of water at 3 days post-treatment. Fish were able to survive in 150 and 250 µl of water with a high rate of survival by 2 days of treatment. However, some death was seen wells containing 150 µl at 2 and 3 days. In contrast, fish survived in wells containing 250 µl at 3 days post-treatment. DMSO had no significant effect on embryo survival.

Thus, for treatment regimes requiring 2 days of analysis, 150 µl of egg water is sufficient for an assay volume. In contrast, for treatments regimes requiring 3 days of analysis, 250 µl of egg water is required.

F. RAG2-GFP thymocyte response to Dexamethasone.

In order to determine if 5 to 8 day old fish are able to absorb chemicals from the water, and if normal GFP-labeled T-cells are responsive to chemical ablation, we treated 5 day-old *RAG2-GFP* fish with varying doses of dexamethasone (DEX). Specifically, 6 GFP-positive RAG2-GFP embryos were arrayed into 6 well plates containing 5 ml of egg water. Wells contained either 0.1% ethanol (control), 250 µg/mL DEX (1% ethanol), 25 µg/mL DEX (0.1% ethanol), 12.5 µg/mL DEX (0.05% ethanol), 5 µg/mL DEX (0.02% ethanol), or 1 µg/mL DEX (0.004% ethanol). Fish were assessed for GFP expression in the thymus at 6, 7, and 8 dpf

G. RAG2-GFP thymocytes are responsive to chemical ablation.

RAG2-GFP fish responded to DEX treatment (Figure 17). Specifically, GFP-positive T-cells were not detected in *RAG2-GFP* embryos treated with 250 µg/mL of DEX at 3 days post-treatment (8 dpf). In contrast, GFP-labeled T-cells were present in fish treated with 0.1% ethanol. Partial responses were seen in DEX-treated fish receiving a 25 µg/mL dose, with 2 of 6 fish showing complete absence of T-cells and several having decreased T-cells in the thymus by 3 days post-treatment.

These results indicated that 5 to 8 day-old zebrafish embryos were able to absorb chemicals from the water, and the normal GFP-labeled T-cells were responsive to chemical ablation by DEX. Finally, these experiments highlight the use of transgenic fish for the development of drug based screens for immunosuppressive agents, and illustrate the ease of screening for chemical suppressors in the fish.

H. A Chemical Screen to identify drugs that inhibit BCL2 function.

Five-day-old *RAG2-EGFP-zBCL2* transgenic zebrafish embryos will be irradiated and three fish arrayed per well into a 96 well plate. Each well will contain 150-250 microliters of egg water containing 8-10 chemical compounds. Fish will be analyzed for expression of GFP in the thymus at 2, and 3 days post-treatment as an indication of T-cell ablation due to apoptosis. Because T-cells in the *RAG2-EGFP-zBCL2* transgenic line are insensitive to irradiation and because most drugs will have no affect on BCL2 function, thymocytes from transgenic fish will remain GFP labeled 2 days post-IR treatment. However, if a chemical is able to inhibit BCL2 function, T-cells will die and by 2 to 3 days post-treatment, thymocytes will be absent (as detected by GFP fluorescence). Drugs combinations that suppress BCL2 function will be tested individually on both *RAG2-EGFP-zBCL2* and *RAG2-GFP* transgenic fish to (1) identify single compounds that inhibit BCL2 function , and (2) control for drugs that ablate T-cell function (*i.e.*, compounds that kill T-cells independent of the transgenic line used).

I. A Dominant Genetic Screen to identify suppressors of BCL2 function.

Our transgenic *RAG2-EGFP-zBCL2* fish will also aid in the understanding of genetic programs that regulate apoptosis in T-cells *in vivo*. Specifically, a genetic modifier screen will be used to assess T-cell regression following irradiation treatment of 5 day-old embryos. Because GFP-positive T-cells in *RAG2-EGFP-zBCL2* fish are resistant to irradiation-induced apoptosis, most fish screened will have GFP-positive T-cells following irradiation at 8 dpf. However, if a genetic modifier of BCL2 function is present in mutant fish, then GFP-positive T-cells will undergo apoptosis following irradiation and T-cells from these fish will be GFP-negative.

For example, the following dominant modifier screen may be used to identify mutations that inhibit BCL2 function (Figure 18). *Wik* male fish will be treated with Ethyl-nitrosourea (ENU) to induce point mutations in their sperm. On average, these ENU-treated males contain 100 point mutations per sperm. These males will be bred to AB strain *RAG2-EGFP-zBCL2* fish. The resulting F_1 progeny will be heterozygous for both the *RAG2-EGFP-zBCL2* transgene, and the mutation will be linked to the *Wik* allele. These F_1 fish will be bred to AB strain *RAG2-EGFP-zBCL2* fish and the resulting F_2 progeny will be scored for sensitivity to irradiation. 5-day-old F_2 fish will be irradiated and assessed for T-cell regression on day 8, as determined by GFP expression in the thymus. Once F_2 mutant fish are identified, which lack GFP-positive T-cells in the thymus following irradiation, the F_1-founder fish will be bred to AB strain *RAG2-EGFP-BCL2* fish, and the resulting progeny will be raised to adulthood. Because these fish carry a dominant mutant allele that is only activated by irradiation and acts by affecting BCL2 expressing cells, the heterozygous mutant fish will be viable. These F_2 fish will be used for mapping the mutation, with the dominant allele segregating with the *Wik* alleles.

Assuming approximately 40,000 genes in the zebrafish genome, the analysis of 400 F_1 mutagenized zebrafish should constitute one haploid genome

equivalent. It will be necessary to analyze 3,000 F_1 fish, which should yield seven-fold coverage of the zebrafish genome.

J. A Recessive Genetic Screen to identify suppressors of BCL2 function.

Our model may also be used to identify recessive mutations that disrupt BCL2 function in T-cells following irradiation. For example, the following recessive modifier screen may be used to identify mutations that inhibit BCL2 function (Figure 19). *Wik* male fish will be treated with ENU to induce point-mutations in their sperm. These males will be bred to AB strain *RAG2-EGFP-zBCL2* fish. The resulting F_1 progeny will be heterozygous for the *RAG2-EGFP-zBCL2* transgene, and the mutation will be linked to the *Wik* allele. Eggs harvested from individual F_1 females will be fertilized with UV-inactivated wild-type sperm. The UV-treatment crosslinks the sperm DNA, so that it will not contribute the genome of the developing zygote. A French press will be used to apply "early pressure" to zygotes within 90 seconds following fertilization, which will disrupt spindle formation during meiosis II and impair the segregation of homologous chromosomes. This will lead to gynogenetic diploid progeny, with 10% to 50% of the offspring being homozygous for mutated alleles. The resulting F_2 embryos will express the *RAG2-EGFP-zBCL2* transgene and be homozygous for mutations. Five day-old F_2 fish will be irradiated and assessed for T-cell regression on day 8, as determined by GFP expression in the thymus. Once F_2 mutant fish are identified that lack GFP-positive T-cells in the thymus following irradiation, the F_0 founder fish will be bred to AB strain *RAG2-EGFP-BCL2* fish, and the resulting progeny will be raised to adulthood. Because these fish carry a recessive mutant allele that is only activated by irradiation, and acts by affecting BCL2 expressing cells, the heterozygous mutant fish will be viable. These F_2 fish will be in-crossed to produce F_3 homozygous mutant fish that contain the *RAG2-EGFP-zBCL2* transgene. The F_3 fish will then be used to map the mutation, with the phenotype (*i.e.* loss of GFP-labeled T-cells following irradiation) segregating mutations linked to the Wik alleles.

K. Discussion.

The zebrafish is a useful system for discovering new small molecule inhibitors of BCL2 or related family members because T-cell regression in transgenic RAG2-fish can be scored as an "all or nothing" response (see above) and because T-cells can be easily visualized *in vivo*. This model offers several advantages for drug screening when compared to cell line based screens. First, drugs are tested *in vivo*. Thus, toxicity and uptake into affected tissues are determined at the time of screening. Second, small molecule inhibitors are added to the water that the fish swim in, which may be used to identify compounds that are water soluble and orally available. Finally, genetic screens using this zebrafish line, in combination with the *RAG2-EGFP-mMYC* transgenic fish, will provide new insight into the pathways that transform T-cells, as well as identification of genetic enhancers and suppressors of BCL2.

EXAMPLE 3: Transgenic Models of High-Grade Astrocytoma

High-grade astrocytomas are the most common and devastating adult brain tumors, spreading so rapidly that patients seldom survive more than 9-12 months. Despite progress in surgical, radiation and chemotherapy technologies, there has been little improvement in the outcome of patients with astrocytoma over the last twenty years. Clearly, novel approaches are needed to better understand the biological basis of this disease before effective therapies can be developed.

The brain consists of two main types of cells, neurons and glia, which multiply and migrate in precise patterns during early development, resulting in the complex structures of the brain. As the brain develops in the embryo, the proliferation and migration of glia is controlled by the coordinated activity of many genes. While some genes are needed to begin normal glial cell proliferation and migration, others are necessary for appropriately stopping these processes. Astrocytomas are cancers specific to glia and in human tumor samples, a

number of genes that normally function during glial cell development are consistently mutated. These mutations result in the loss of control of normal cell proliferation and lead to tumor formation. Genes that control cell proliferation during development, and keep it in check throughout life, are called tumor suppressors. In astrocytoma, mutated tumor suppressor genes include *p53* (seen in 60-70% of all cases), and two genes in a region of human chromosome 10q (75-90% of all cases): *PTEN* and MXI1. Other genes are not mutated, but are inappropriately turned on in these tumors, such as the *EGFR* gene (50% of all cases). A combination of these genetic events is necessary to produce a malignant high-grade astrocytoma, adding to the complexity of the disease and its treatment.

Zebrafish are advantageously used to study astrocytoma because zebrafish brains form much like humans. Not only do the same genes, including those listed above, function during its development, but they can also develop brain tumors. The zebrafish offers many other advantages including: fecundity, each female laying 2-300 eggs/week, and transparent embryos that develop outside of the mother. This allows the direct observation of the rapidly developing brain, which is well formed by 48 hours. Zebrafish may be created which specifically misexpresses genes, such as *EGFR*, in glial cells by directing gene expression using a glial-specific promoter, such as, *GFAP* (glial fibrillary acidic protein). As was done for in the experiments outlined in Example 1, engineered fish that express green fluorescent protein in their glia could allow for the direct observation of the transformed glial cells in the transparent, living animal expressing the *GFAP-EGFR* transgene. Other genes and promoters may be used to develop new models of high-grade astrocytomas.

Astrocytoma transgenic fish lines may be used in additional mutagenesis and chemical studies to identify genes and other test drugs and agents that may suppress or modify the disease in these animals (as described more fully in Example 1). This information may then be used in the development of effective treatments for this disease.

EXAMPLE 4: Transgenic Models of Rhabdomyosarcomas

Rhabdomyosarcomas are a heterogeneous group of malignant tumors and are the most common soft-tissue sarcoma in children of 15 years or younger. Rhabdomyosarcoma consists of two histologic subtypes, alveolar and embryonal, each characterized by the misexpression of different subsets of genes. Alveolar rhabdomyosarcoma expresses members of the myogenic regulatory (*MRF*) family of transcription factors, normally found in developing skeletal muscle, and PAX3 and *PAX7* genes, members of the Paired Box transcription factor family. In nearly all alveolar rhabdomyosarcoma cases, these *PAX* genes are found fused to the forkhead family member gene (*FKHR*) due to specific translocations, notably t(2;13)(q35;q14) comprising 95% of cases, and a variant, t(1;13)(p36;q14) in 5% of cases. The resulting fusion proteins are thought to activate downstream targets of *PAX3* and *PAX7*, leading to cell transformation. These translocations are often accompanied by the over-expression of genes such as *MYCN*, *MDM2*, *CDK4* and *GLI1*. The molecular basis of the embryonal subtype, the predominant form of rhabdomyosarcoma, remains unclear, but is thought to be characterized by loss of heterozygosity (LOH 11p15) and the disruption of other genes such as *IGF2*, *ATR*, *ATM*, *PTEN*, *PTC, pRB, p16* and *TP53*.

The aggressive nature of these tumors makes their effective treatment particularly difficult. While rhabdomysarcomas can be observed in genetically-engineered, mammalian disease models, they are often associated with other tumor types. While informative, a more specific model of rhabdomyosarcoma is necessary to elucidate its molecular basis and to identify novel genes that may ultimately be used as targets for the development of novel therapeutic strategies.

Zebrafish develop soft-tissue tumors, both spontaneously and in response to embryonic exposure to mutagens and tissue specific promoters such as *MyoD* and *alpha-actin* have been identified, both of which drive strong expression of *GFP* in developing muscle.

The specific translocations that typify alveolar rhabdomyosarcoma result in the generation of PAX3-FKHR and PAX7-FKHR fusion proteins. Incorporating these fusion genes, and/or the other genes expressed in rhabdomyosarcoma such as *MYCN, MDM2, CDK4* and *GLI1,* into the *MyoD* and *alpha-actin* promoter constructs will be useful as a model of rhabdomyosarcoma in the zebrafish.

Alternatively, the *PAX3-FKHR* and *PAX7-FHKR* genes may be fused with *GFP* in order to directly visualize transgene expression *in vivo* throughout early zebrafish development. The ability to directly visualize the transgene-GFP expression *in vivo* throughout zebrafish maturation will facilitate the identification and analysis of the tumorigenic phenotype.

Although a zebrafish disease model based upon the fusion proteins found in alveolar rhabdomyosarcoma-associated translocations is probably the most effective way to phenocopy rhabdomyosarcoma, it is possible that the expression of other genes known to be over-expressed in this disease may be necessary to observe tumorigenesis. Therefore, additional transgenic lines will be generated using the muscle-specific promoter constructs, such as those described herein, to drive the expression of individual oncogenes such as *MYCN, MDM2, CDK4, GLI1* or a dominant negative form of p53, which have all been shown to be associated with rhabdomyosarcoma. Furthermore, once such transgenic lines are established, they may be tested for the ability to genetically interact with each other by intercrossing them and analyzing their phenotype for synergistic or complementing effects, as described in Example 1 and elsewhere herein.

The development of transgenic models of rhabdomyosacrcoma, as well as other types of cancer, will be useful to exploit the forward genetics capacity of this vertebrate model system to identify genes that regulate rhabdomyosarcoma development. Once transgenic lines giving rise to the rhabdomyosarcoma phenotype are generated, they may be used as the basis for an ethyl-nitrosurea

(ENU) mutagenesis screen, as described herein. Briefly, male transgenic fish may be treated with, *e.g.,* ENU to induce point mutations in germ cells, and then mated with transgenic female fish, giving rise to F_1 progeny that are heterozygous for potential mutations. The eggs are collected from mature F_1 females, fertilized with UV-inactivated sperm and undergo 'early pressure'. This results in gynogenetic diploid animals with up to 50% of the offspring being homozygous for alleles carrying mutations that may result in an alteration in the rhabdomyosarcoma phenotype normally seen in the transgenic line. Once potential mutants are identified in the progeny of a particular F_1 female, she will be outcrossed to recover a line of heterozygous mutants. These fish may be incrossed and mutant homozygous progeny identified. The mutations may then be genetically mapped to specific linkage groups, such as by using a genome-wide scanning approach. Once a linkage group is determined, polymorphic markers flanking the mutated gene may be identified and other methods well known to those skilled in the art may be used to initiate positional cloning using the physical radiation hybrid map. This process will lead to cloning of the mutated gene by direct sequencing and candidate gene analysis. As in Example 1, numerous types of genetic screens may be designed including, but not limited to, dominant modifier screens, homozygous early pressure screen, or homozygous F_3 screens, which are well known to those of skill in the art. The mutagen may be, for example, ENU, EMS, or viral insertion.

The genes identified as a result of this zebrafish disease model represent potential regulators of the molecular-genetic pathway leading to rhabdomyosarcoma and may then be used to identify human homologs. Using this method, novel targets for drug design and developing improved therapies to treat this devastating disease may be determined. Similarly, chemical based screens may identify therapeutic agents that can suppress the phenotypes associated with overexpression of oncogenes in muscle cells. Such chemicals may then be candidates for clinical trials in human patients suffering from rhabdomyosarcoma.

EXAMPLE 5: Transgenic Models of Acut My loid L ukemia

The mutations and gene rearrangements commonly seen in acute myeloid leukemias typically result from a chromosomal translocations such as the t(8;21) or t(15;17), generate chimeric oncoproteins by fusing one or two transcription factors (Look, 1997). However, these alterations are not sufficient to explain the induction of acute leukemia. For example, expression of PML-RARα in murine myeloid cells causes leukemia only after a long pre-leukemic phase (He *et al.*, *Proc Natl Acad Sci USA*, *94*:5302-5307 (1997) and Grisolano *et al.*, *Blood 89*:376-387 (1997)) indicating that other mutations must occur, particularly affecting pathways involved in apoptosis and cell cycle control. It is now known that activated FLT3 kinase mutations occur in a high percentage of APL patients, possibly providing a second transforming event. FLT3-activating mutations occur in approximately 25% of cases of AML, making them among the most frequent mutations linked to the molecular pathogenesis of human AML. These mutations are either internal tandem repeats (ITD) that occur in the juxtamembrane domain of the FLT3 receptor or point mutations in the negative regulatory loop domain of the kinase (Yamamoto *et al., Blood 97*:2193A (2001)) that causes constitutive activation of FLT3. Although *in vitro* cell lines and murine models are being developed to study FLT3 activation, the forward genetic capacity of the zebrafish system provides a unique advantage in that it permits the unbiased detection of mutations in many potentially novel genes that lead to leukemia. Recent studies have indicated that the spatio-temporal expression of zebrafish homologues of known mammalian myelopoietic genes and the morphologic and cytochemical features of zebrafish myelopoiesis are remarkably conserved (Bennett *et al., Blood 98*:643-651 (2001), Herbomel *et al. Development* 126:3735-3745 (1999), reviewed in Hsu *et al., Current Opinion in Hematol, 81*:245-251 (2001)).

Studies in the mouse indicate that overexpression of the *FLT3-ITD* mutated cDNA causes a myeloproliferative disorder (Kelly *et al., Blood, 99*:310-318 (2002)). Furthermore, studies of other tyrosine kinase oncogenes in the

mouse, such as BCR/ABL, have demonstrated a lack of differentiation block, more active proliferation, and strong anti-apoptotic activity (Evans *et al.*, *Cancer Res*, *53*: 1735-1738, (1993); Laneuville *et al.*, *Cancer Res.*, *54*:1360-1366 (1994); Druker *et al.*, *Nat Med*, 2: 561-566 (1996); and Carlesso *et al.*, *Oncogene* *9*: 149-156 (1994)). Numerous zebrafish promoters have been identified that drive strong expression of *GFP* in myeloid cells, as described herein, including the *PU.1, MPO, or C/EBPα* promoters. Utilizing stable lines of transgenic zebrafish expressing a constitutively activated *FLT3, BCR/ABL*, and *PML-RARα* allows for the study of acute myeloid leukemia in the zebrafish, with *FLT3* overexpression in myeloid cells resulting in hypercellular kidney marrow, splenomegaly, and neutrophilia resembling a myeloproliferative disorder. Enhancer-suppressor screens, as described herein, will in identifying specific modifier genes that encode targets for the development of small molecule inhibitors that can be used as therapy for clonal myeloid neoplasias.

EXAMPLE 6. Conditional models of cancer

Many of the underlying mechanisms that lead to neuorendocrine carcinoma, pancreatic carcinoma, ovarian carcinoma, testicular carcinoma, stomach cancer, colon cancer, renal cancer, melanoma and acute or chronic myeloid leukemia have yet to be fully understood. Identifying the genes mutated in these diseases will lead to new insights into cancer as a whole. Additionally, using a vertebrate model system in which genetic or chemical suppressors can be identified that inhibit or delay disease progression will be necessary to identify new drug targets for the development of targeted chemotherapies.

As described in Example 1, conditional transgenic zebrafish lines may be produced using a CRE/Lox-mediated excision event to drive expression of the *EGFP-mMYC* transgene, and CRE-mediated recombination was shown to be active in zebrafish. However, use of a site-specific recombination strategy may be more broadly applicable to developing zebrafish models of a variety of other cancers, including but not limited to: neuorendocrine carcinoma, pancreatic

carcinoma, ovarian carcinoma, testicular carcinoma, stomach cancer, colon cancer, renal cancer, melanoma and acute or chronic myeloid leukemia.

One strategy may be used to create stable transgenic fish models of cancer in which an oncogene is regulated in any tissue by using a regulatable site-specific recombinase. A transgenic fish whose genome has stably-integrated therein a transgene cassette comprising a Floxed, Loxed or FRTed reporter gene, such as a fluorescent protein gene (*e.g., GFP, RFP, BFP, YFP*, or *dsRED2*) or a luciferase gene, which comprises at strong stop-site, is regulated by a ubiquitous gene promoter (*e.g., beta-actin* or *EF1-alpha*), and a second transgene (*e.g.,* an oncogene) placed immediately after the reporter gene. The second gene product is adjacent the reporter gene, but is not expressed in the absence of recombinase activity because of the strong transcription stop-site within the reporter gene. A second transgenic fish whose genome has stably integrated therein a *flip* (for use with FRT) or *Cre-recombinase* (for use with Flox or Lox) gene operably linked to a heat shock inducible promoter (*e.g., HSP-70*) is mated to the first transgenic fish. Cells within the offspring are then activated by heating with a laser, as described elsewhere herein. Once specific cells are heated with the laser, recombination is activated causing excision of the fluorescent protein gene and juxtaposition of the ubiquitous gene promoter adjacent to the oncogene. This results in the activation of the oncogene in specific cells and may be targeted to many different T-cell types.

Previous experiments have shown that the *HSP-70* promoter is strictly regulatable in transgenic zebrafish by laser-activation of individual cells (Halloran *et al.*, 2000) and that the *HSP-70* promoter can drive expression of genes other than *GFP* in transgenic animals (Xiao *et al.*, 2003). Individual cells can be identified easily because the fish is translucent in development. Additionally, the fish develop most organ systems by 5 dpf, and therefore, nearly all cell types can be targeted by laser-activation. Because this strategy relies on the generation of a transgenic fish in which oncogene activation can be targeted to any cell in the body (*i.e.*, the use of a ubiquitous promoter driving expression of the reporter

gene and the second oncogene), new models of cancer in the fish will be able to be developed, which do not require the generation of new transgenic lines with each cancer being studied.

For example, creation of transgenic zebrafish which expresses the loxed-*dsRED* allele adjacent the *EGFP-mMYC* transgene, which is regulated by the *beta-actin* promoter (*β-actin-Lox-dsRED-Lox-EGFP-mMYC*) may be useful for generating models to study pancreatic beta cell tumors, invasive islet adenocarcinoma, Burkitt's Lymphoma, Acute Myeloid Leukemia, colon carcinoma, glioblastomas, and melanoma all of which have activation of the *MYC* oncogene. Development of each of these models only requires activation of CRE within the cells that give rise to affected tissues, which is facilitated by laser activation described herein.

While the inventions have been illustrated and described in detail in the drawings and foregoing description, the same is to be considered as illustrative and not restrictive in character, it being understood that only the preferred embodiments have been shown and described and that all changes and modifications that come within the spirit of the invention are desired to be protected. In addition, all references cited herein are indicative of the level of skill in the art and are hereby incorporated by reference in their entirety.